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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 8191-PA01

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR): 09/856655

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/AU99/01052	25 November 1999	25 November 1998

TITLE OF INVENTION
IMMUNOSTIMULATORY DNA

APPLICANT(S) FOR DO/EO/US
STACEY, Katryn et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☒ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☒ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
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Date of Deposit MAY 24, 2001

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington D.C. 20231

Jeanne Brush
(Name)

(Signature)

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR) 09/856655	INTERNATIONAL APPLICATION NO. PCT/AU99/01052	ATTORNEY'S DOCKET NUMBER 8191-PA01

24. The following fees are submitted:.		CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :			
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	$1000.00		
☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	$860.00		
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☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)	$690.00		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)	$100.00		
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Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).		$130.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	20 - 20 =	0	x $18.00	$0.00	
Independent claims	6 - 3 =	3	x $80.00	$240.00	
Multiple Dependent Claims **(check if applicable).**			☒	$270.00	
TOTAL OF ABOVE CALCULATIONS =				$1,500.00	
☒ Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				$750.00	
SUBTOTAL =				$750.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).			+	$0.00	
TOTAL NATIONAL FEE =				$750.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐				$0.00	
TOTAL FEES ENCLOSED =				$750.00	
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a. ☒ A check in the amount of **$750.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **02-4070** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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REGISTRATION NUMBER

24 May 2001
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TITLE

"IMMUNOSTIMULATORY DNA"

FIELD OF THE INVENTION

THIS INVENTION relates to methods for identifying immunostimulatory DNA, which DNA stimulates cells of a mammalian immune system. This invention is also directed to methods for identifying agonists and antagonists of immunostimulatory DNA.

BACKGROUND OF THE INVENTION

A fundamental attribute of the mammalian immune system is the ability to distinguish self from non-self. This is most generally understood with regard to proteins and peptides, but it also applies to DNA.

In this regard, certain cells of the immune system such as B lymphocytes and macrophages are able to distinguish bacterial DNA from vertebrate and plant DNA (Messina *et al.*, 1991, J. Immunol. **147** 1759; Yamamoto *et al.*, 1992, Immunol. **36** 983; Stacey *et al.*, 1996, J. Immunol. **157** 2116; Klinman *et al.*, 1996, Proc. Natl. Acad. Sci. USA **93** 2879). That is, bacterial DNA can stimulate cells of the immune system, and as such is an example of "immunostimulatory DNA".

A core motif common to immunostimulatory DNA is the CpG dinucleotide motif, which appears to be central to the immunostimulatory capacity of DNA (Krieg *et al.*, 1995, Nature **374** 546). However, it is also clear that flanking sequence can be important, in that CpG sequences flanked by a cytosine (C) or guanine (G) nucleotide are less immunostimulatory (Krieg *et al.*, 1995, *supra*).

CpG sequences are relatively common in bacterial DNA, and are generally unmethylated. In contrast, CpG sequences occur less commonly in vertebrate DNA (about 25% of what would be expected based on random base utilization) and are generally methylated (Bird, 1987, Trends Genet. **3** 342; Bird, 1993, Cold Spring Harbor Symp. Quant. Biol. **58** 281), this characteristic being referred to as CpG suppression". Thus, by virtue of the presence of unmethylated CpG sequences, bacterial DNA can

be distinguished by the immune system as being non-self, whereas "CpG suppressed" vertebrate sequences are treated as self. It should also be noted that unmethylated vertebrate CpG sequences tend to be flanked by C or G nucleotides, rendering them less immunostimulatory.

In B lymphocytes, CpG-containing DNA is a polyclonal activator of mitogenesis and cytokine production, and furthermore acts to block apoptosis, such as induced by anti-IgM treatment (Krieg *et al.*, 1995, *supra*; Messina *et al.*, 1991, *supra*; Sun *et al.*, 1997, J. Immunol. **159** 3119; Piseteky & Reich, 1993, Mol. Biol. Rep. **18** 217; Liang *et al.*, 1996, J. Clin. Invest. **98** 1119).

In macrophages and dendritic cells, and indirectly in NK cells, CpG-containing DNA partially mimics the action of bacterial lipopolysaccharide (LPS) (Chace *et al.*, 1997, Clin. Immunol. Immunopathol. **84** 185; Cowdery *et al.* 1996, J. Immunol. **156** 4570; Halpern *et al.*, 1996, Cell. Immunol. **167** 72; Sparwasser *et al.*, 1997, Eur. J. Immunol. **27** 1671; Sparwasser *et al.*, 1997, Nature **386** 336; Stacey *et al.*, 1996, *supra*; Sweet *et al.*, 1998, J. Interferon Cytokine Res. **18** 263; Yamamoto *et al.*, 1992, *supra*). However, there are important differences between the effect of LPS and CpG-containing DNA. For example, although CpG DNA is as effective as LPS at inducing the transcription factor NF-κB and TNF-α mRNA, unlike LPS it is a poor inducer of interleukin 1 β (IL-1β) and does not, by itself, induce nitric oxide synthase (iNOS) (Stacey *et al.*, 1996, *supra*; Sweet *et al.*, 1998 *supra*). It has also been noted that CpG DNA is an excellent inducer of interleukin 12 (IL-12), and thereby acts to promote interferon gamma (IFN-γ) production in mixed cell cultures (Chace *et al.*, 1997, *supra*; Cowdery *et al.*, 1996, *supra*). IFN-γ in turn primes macrophages to respond to immunostimulatory DNA by expressing iNOS, thereby creating a self-amplifying loop with NK cells (Sweet *et al.*, 1998, *supra*).

The emerging consensus is that immune modulation by immunostimulatory DNA involves elicitation of IL-12 production by macrophages or dendritic cells which thereby contributes to the polarization

of T-lymphocyte responses towards an IFN-γ-producing T-helper 1 (Th1) phenotype (Carson & Raz, 1997, J. Exp. Med. **186** 1621; Chu *et al.*, 1997, J. Exp. Med. **186** 1623; Davis *et al.*, 1998, J. Immunol. **160** 870; Lipford *et al.*, 1997, Eur. J. Immunol. **27** 2340; Roman *et al.*, 1997, Nature Med. **3** 849; Weiner *et al.*, 1997, Proc. Natl. Acad. Sci. USA **94** 10833; Zimmerman *et al.*, 1998, J. Immunol. **160** 3627). As would be expected from the Th1 cytokines induced by immunostimulatory DNA, the immune response is typically Th1-like, and characterized by a predominance of IgG_{2a} antibody production and strong CTL induction. In fact, it has been noted that CpG-containing DNA is a stronger Th1 adjuvant than complete Freunds adjuvant (Chu *et al.*, 1997, *supra*).

An important consequence of the immune response to immunostimulatory DNA is that when such sequences are present in a plasmid vector, the plasmid vector can effectively act as an adjuvant. This has implications for vectors used in DNA vaccines, and it has been reported that CpG sequences are essential for effective DNA vaccination (Gregoriades, 1998, Pharm. Res. **15** 661, Klinman *et al.*, 1997, J. Immunol. **158** 3635; Sun *et al.*, 1988, J. Exp. Med. **187** 1145; Sato *et al.*, 1996, Science **273** 352). It will also be appreciated that an increasing number of studies have utilized synthetic oligonucleotides (ODN) which contain immunostimulatory CpG sequences as immunomodulators in their own right (Schwartz *et al.*, 1997, J. Clin. Invest. **100** 68; Wooldridge *et al.*, 1997, Blood **89** 2994; Kline *et al.*, 1998, J. Immunol. **160** 2555).

The limited mechanistic studies of how immunostimulatory DNA stimulates macrophages and B lymphocytes suggest that immunostimulatory DNA must be internalized. For example, chloroquine which interferes with endosomal function, blocks the actions of immunostimulatory DNA, but not the actions of LPS (MacFarlane & Manzel, 1998, J. Immunol. **160** 1122; Yi *et al.*, 1998, J. Immunol. **160** 4755). Thus, it has been postulated that cellular recognition of immunostimulatory DNA probably occurs within the cytoplasm or nucleus.

Replication protein A (RPA) is a known DNA-binding activity in eukaryotic cells.

RPA exists as a complex of three subunits:-

(i) a 70 kD subunit (RPA70 or RPA1) which contains two DNA-binding domains;

(ii) a 34 kD subunit (RPA34 or RPA2); and

(iii) a 14 kD subunit (RPA14 or RPA3).

RPA34 and RPA14 have been shown recently to form a separate DNA binding domain that may also interact with the C-terminus of RPA70.

The major functional role for RPA is as an indispensable player in normal cellular DNA replication, namely by being required for DNA strand separation and unwinding. (reviewed in Wold, 1997, Ann. Rev. Biochem. **66** 61). RPA also recognizes DNA damaged by agents such as UV light and cisplatin, and contributes to repair of damaged DNA (Burns *et al.*, 1996, J. Biol. Chem. **271** 11607; Patrick & Turchi, 1998, Biochemistry **37** 8808). Thus, the crucial role of RPA in general processes relating to cellular DNA replication and RPA's relative lack of sequence specificity, had rendered RPA an unlikely candidate as a sequence-specific receptor for CpG-containing immunostimulatory DNA (Wold, 1997, *supra*).

OBJECT OF THE INVENTION

In contrast to the expectations provided by the prior art, the present inventors have identified RPA as a cellular receptor for immunostimulatory DNA. This discovery has provided new molecular and cellular methods of identifying immunostimulatory DNA.

It is therefore an object of the invention to provide a method of identifying an immunostimulatory DNA.

SUMMARY OF THE INVENTION

The present invention is broadly directed to identifying immunostimulatory DNA. The methods of the invention include detection of complexes formed between RPA subunits and immunostimulatory DNA, and

detection of macrophage responses to immunostimulatory DNA, such responses including CSF-1R down-regulation and cell cycle arrest. These methods also enable identification of immunostimulatory DNA agonists and antagonists.

In a first aspect, the present invention provides a method of detecting immunostimulatory DNA, including the steps of:-

(i) combining isolated RPA with a sample containing DNA;

(ii) forming a complex between said isolated RPA and immunostimulatory DNA, if present in said sample; and

(iii) detecting said complex formed at step (ii).

In a second aspect, the invention provides a method of identifying an immunostimulatory DNA agonist or antagonist, including the steps of:-

(i) combining isolated RPA with a sample suspected of containing an immunostimulatory DNA agonist or antagonist; and

(ii) determining whether or not a complex forms between said isolated RPA and said agonist or antagonist, formation of a complex indicating that said agonist or antagonist is present in said sample.

In a preferred embodiment, an immunostimulatory DNA is added at step (i) so that at step (ii) said agonist or antagonist prevents formation of a complex between said immunostimulatory DNA and said isolated RPA.

Preferably, the complexes formed at step (ii) are detected by EMSA.

More preferably, an anti-RPA antibody is included at step (i).

In a third aspect, the invention provides a kit for detecting immunostimulatory DNA, said kit comprising:-

(i) one or more isolated RPA subunits; and

(ii) an RPA-specific antibody.

In a fourth aspect, the invention provides a method of detecting an immunostimulatory DNA including the steps of:-

(i) combining macrophage cells with a sample suspected of containing immunostimulatory DNA;

(ii) measuring a response by said macrophage cells to said immunostimulatory DNA if present in said sample, said response selected from the group consisting of:-

(a) a cell-cycle arrest; and

(b) a reduction in CSF-1 receptor (CSF1-R) expression.

In a fifth aspect, the invention provides a method of identifying an immunostimulatory DNA antagonist, including the steps of:-

(i) combining macrophage cells with immunostimulatory DNA and a sample suspected of containing an immunostimulatory DNA antagonist;

(ii) measuring a response, or an absence of said response, by said macrophage cells to said immunostimulatory DNA, said response selected from the group consisting of:-

(a) a cell-cycle arrest;and

(b) a reduction in CSF-1 receptor (CSF1-R) expression;

wherein said absence of a response is indicative of said antagonist being present in said sample.

In a sixth aspect, the invention provides a method of identifying an immunostimulatory DNA agonist, including the steps of:-

(i) combining macrophage cells and a sample suspected of containing an immunostimulatory DNA agonist;

(ii) measuring a response by said macrophage cells selected from the group consisting of:-

(a) a cell-cycle arrest;

(b) a reduction in CSF-1 receptor (CSF1-R) expression;

wherein a response by said macrophage cells is indicative of said agonist being present in said sample.

In a seventh aspect, the present invention provides an immunostimulatory DNA, agonist or antagonist identified by the methods of the invention.

In an eighth aspect, the present invention provides an immunostimulatory DNA antagonist comprising a DNA having a phosphorothioate backbone.

Preferably, the immunostimulatory DNA is a CpG-containing DNA having a phosphodiester backbone.

In a ninth aspect, the present invention provides therapeutic or prophylactic compositions which comprise the immunostimulatory DNA, agonist or antagonist of the seventh aspect or eighth aspect, together with an acceptable carrier or delivery agent.

Throughout this specification, unless the context requires otherwise, "comprise", "comprises" and "comprising" are used inclusively rather than exclusively, in that a stated integer or integer group may include one or more other non-stated integers or groups of integers.

It will also be appreciated that scientific terms are to be given their usual scientific meaning, although in some cases certain terms will be defined in order to assist interpretation by the skilled person.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1

Effect of various oligonucleotides on luciferase activity in RAW264a4 cells. The indicator RAW264a4 cells with integrated HIV-LTR-luciferase construct were cultivated with the indicated oligonucleotides (defined in Materials and Methods) at the concentrations shown. After 2 hrs, or the indicated time in panel C, they were washed with PBS, lysed and luciferase activity was determined. In panel A and B the luciferase activity of control cells is

normalised to a value of 1. Results are the average of triplicate determinations. In Panel C, RLA is relative luciferase activity.

FIG. 2

Effect of oligonucleotides on TNF-α secretion. RAW264 cells (5×10^5 in 0.5 ml in a 24 well plate) were incubated with the oligonucleotides shown or with LPS as a positive control at the concentrations indicated for 18 hrs. The supernatants were harvested and the level of TNF-α secretion was assessed by ELISA. Results are the average of duplicates.

FIG. 3

Uptake of propidium iodide as an assay of DNA action. 10^6 BMM were harvested, washed and replated for 24 hrs in the presence of CSF-1, or in its absence (lower three panels). As indicated, *E. coli* genomic DNA (10 µg/ml) or LPS (100 ng/ml) was added. After 24 hrs, propidium iodide was added for 1.5 hrs as described in Materials and Methods. The FACS analysis plots fluorescence (vertical axis) against forward light scatter (an indicator of cell size). Cells in the R2 region of the profile are assessed as having taken up propidium iodide and therefore are non viable. Note that CSF-1, DNA and LPS all repress the appearance of these cells and also maintain a larger average (indicated by the right shift on the horizontal axis).

FIG. 4

LPS and bacterial DNA cause cell cycle arrest in proliferating macrophages. BMM (1×10^6) were incubated in 10^4 U/ml CSF-1 with addition of LPS (100 ng/ml) or *E. coli* genomic DNA (10 µg/ml) as indicated for 24 hrs. They were then permeabilized and stained for DNA content using propidium iodide as described in Materials and methods. Cells are ascribed a position in G_1, G_2M or S phase based upon 2N, 4N or intermediate DNA content respectively as indicated.

FIG. 5

Down modulation of CSF-1 surface receptor expression in bone marrow-derived macrophages. A. Bone marrow-derived macrophages were harvested, washed and replated on glass cover slips in the presence or

absence of 10^4 U/ml CSF-1 overnight. CSF-1-starved cells were incubated with CSF-1, *E. coli* genomic DNA (10 μg/ml) or LPS (100 ng/ml) as indicated, the fixed for immunofluorescence localisation of CSF-1R antigen as described in Materials and Methods. Note the high level of surface CSF-1R expression in CSF-1 starved cells, which is rapidly down-modulated in response to CSF-1, DNA or LPS. B. FACS profiles CSF-1R down modulation. Bone marrow-derived macrophages were starved of CSF-1 overnight, and incubated for 1 hr without or with CSF-1 (10^4U/mL), *E. coli* DNA (ECDNA; 10 μg/mL), LPS (100 ng/mL), DNAse I treated *E. coli* DNA (DNASED EC DNA; 10 μg/mL), calf thymus DNA (CTDNA; 10 μg/mL) or salmon sperm DNS (SSDNA; 10 μg/mL) for 1 hr. Cells were harvested and stained for CSF-1R surface expression and then analyzed by flow cytometry. C. Time course analysis of CSF-1 treated and *E. coli* genomic DNA treated bone marrow-derived macrophages (BMM). BMM were starved of CSF-1 overnight, and incubated without or with CSF-1 (10^4U/mL) or *E. coli* DNA (ECDNA; 10 μg/mL) for varying times as indicated and then analyzed by flow cytometry. D. Time course analysis of AO-1 and NAO-1 treated BMM.MM were starved of CSF-1 overnight, and incubated without or with 20 μg/mL CpG-containing AO-1, or 20 μg/mL GpC-containing NAO-1 for indicated times and analyzed by flow cytometry.

FIG. 6

Electrophoretic mobility shift analysis of location and specificity of ssDNA binding activity in various extracts. Radiolabelled single-stranded (AO-1) or double-stranded ODN probes (PU-1, the PU box recognition sequence or Octamer; see Materials and Methods) were incubated with 2 μg of cytoplasmic (C) or nuclear (N) extracts in a binding buffer containing 12% glycerol, 20 mM HEPES (pH 7.9) plus 0.5 mM dithiothreitol, 2 mM EDTA, 40 mM KCl and 20 μg/ml sheared salmon sperm DNA. Extracts were then separated on non-denaturing 7.5% PAGE and exposed to autoradiographic film. The unbound probe has been run off the end to increase resolution of the protein bands. Panel A demonstrates the compartmentation of binding

activity for ssAO-1 compared to well documented targets for dsDNA binding proteins in macrophages; PU.1 and Oct-1. The abundant band towards the bottom of the gel in both the PU.1 tracks (Panel A) has been shown previously to be PU.1, whilst the abundant nuclear band in the Oct tracks is Oct-1. Panel B compares the distribution of activities in RAW264 and NIH-3T3 fibroblasts. In both panels A and B the cold competitor is 20 µg/ml of sheared salmon sperm DNA. Panel C shows cytoplasmic extracts of RAW264 cells incubated with labelled ssAO-1 ODN. Various amounts of salmon sperm DNA are used as a cold competitor DNA. Note there is some selective loss of the B' band at 0.2 µg of added cold competitor (20 µg/ml). In Panel D RAW264 cytoplasmic extracts were incubated with labeled immunostimulatory ssODN probe M12 together with plasmid DNA (the reporter plasmid pGL3C) in excess (fold-excess on a µg per µg basis) as indicated. Note the selective release of the lower band (band C) which has resolved slightly further in this particular experiment compared to panels A-C.

FIG. 7

Electrophoretic mobility shift analysis of sequence specificity of ssDNA binding activity. RAW264 cytoplasmic extracts were incubated with radiolabelled probes as indicated and separated on 7.5% non-denaturing PAGE. Buffer conditions were changed in different experiments to optimise visualisation of particular bands as indicated. Panel A compares the binding of RAW264 cytoplasmic proteins to single stranded or double-stranded immunostimulatory ODN AAC-22. Binding buffer is the same as the nuclear extract buffer D and to avoid loss of dsDNA binding activities, no cold competitor was added. Four independent extracts of RAW264 (#1 to #4) show the same pattern of binding and the pattern is similar whether the probe is single-stranded (left panel) or double-stranded (right panel). Cold competition with single-stranded AAC-22, reveals that a 125-fold excess of self ODN completely prevents binding of both ss and ds-AAC-22, whereas the same amount of ss non-stimulatory ACC-22 failed to prevent

significant binding of ss or dsAAC-22. Panel B shows quantitative cold-competition of binding of ss stimulatory ODN AAC-22 with cold self ODN or non-stimulatory ACC-22 ODN. Note that there is approximately 4-fold difference in apparent relative affinity for stimulatory compared to non-stimulatory ODN. Binding buffer is also Buffer D. Panel C compares a different set of ODNs using a separate RAW264 cytoplasmic extract, in this case band C (the lower band) is particularly abundant. Labelled probes are the immunostimulatory ODN AO-1 and the non-stimulatory GC transversion NAO-1 or the methylated form AOM-1. Note that in each case the pattern of binding is the same, but regardless of which ODN is labelled, AO-1 is the more effective cold-competitor. Binding buffer is the same as in FIG. 6, without cold competitor. This pattern is confirmed independently in panel D, with a separate RAW264 extract and AO-1 labelled as indicated. In this case, salmon sperm DNA was added (20 µg/ml) but does not alter the pattern of relative binding activity. Note that AO-1 is at least 5 fold more effective as cold competitor than either NAO-1 or AOM-1. In Panel E, the labelled probe is again AO-1 and the cold competitors are a separate immunostimulatory ODN (1668) and the weak activator IL-12p40. Note that a 10-fold excess of 1668 reduces binding to a similar extent to a 50-fold excess of IL-12p40 ODN. Binding buffer in this case contains 10 mM KCl, 5 mM $MgCl_2$ and 50 mM $CaCl_2$ to optimise detection of band C.

FIG. 8

Characteristics of the binding of ssDNA by RAW264 cytoplasmic proteins. Panel A shows a further independent confirmation of the ability of stimulatory ODN (AO-1) to cold-compete approximately 5-fold more effectively than non-stimulatory ODN, NAO-1. Binding buffer is the same as in FIG. 6 with 20 µg/ml salmon sperm DNA as competitor. In the right panel, the labelled ODN was allowed to preincubate with cytoplasmic extract for 15 mins at room temperature prior to addition of cold competitor. Note that the faster migrating band is still competed but no reduction in the slower band (band A) is evident. Panel B examines the effect of changing the ion

composition of the binding buffer. In each case the buffer is the and incubations are carried out in 20 mM HEPES buffer pH 7.9, varying only by additions of $MgCl_2$ or KCl as indicated. Note that binding of band A. the slower band, is increased by KCl and decreased by $MgCl_2$. Note also that the middle band, band B, is detected most readily in the presence of $MgCl_2$ and the absence of KCl.

FIG. 9

Primary gel shifts were carried out in binding buffer as in FIG. 6 and UV cross-linked *in situ* as described in Materials and Methods. The relevant bands were located, excised, extracted and re-run on SDS-PAGE with molecular weight standards as indicated. Bands and UV cross-link times are indicated as follows: Lane 1= Bands A, B and C (0 minutes); Lane 2= Band C (2 minutes); Lane 3= Band B(2 minutes); and Lane 4= Bands A, B abd C (2 minutes).

FIG. 10

Cytoplasmic extracts were prepared from RAW264 cells and from the human macrophage cell line MonoMac6. Prior to addition of labeled ODN, the cytoplasmic extracts were incubated for 10 mins in irrelevant monoclonal antibody, anti-RPA34 or anti-RPA70 monoclonal antibody. Note that anti-RPA34 generates a new supershifted complex and completely represses the formation of the faster-migrating complexes. Anti-RPA70 abolishes the formation of the faster-migrating complexes in the human line only, but does not generate a super-shifted band. Binding buffer in this case contained 20 mM HEPES pH 7.5, 140 mM KCl, 13 mM NaCl, 5 mM $MgCl_2$ and 20 µg/ml salmon sperm DNA.

FIG. 11

Phosphorothioate-modified CpG ODN are more active in induction of nitrite production than sequence identical phosphodiester ODN. Bone marrow derived macrophages (Panel A) or RAW264 cells (Panel B) were primed with interferon-γ, then incubated for a further 24hrs with the indicated concentration of PS (AOS-1, NAOS-1) or PO (AO-1) prior to harvesting and

measurement of nitrite as described in Materials and Methods. Points are the average of triplicates. The experiment is representative of three.

FIG. 12

Phosphorothioate-modified CpG ODN are more active than phosphodiester ODN in maintenance of viability of CSF-1-starved bone marrow-derived macrophages (BMM). BMM were starved of CSF-1 for 48hrs with no additions or the indicated concentrations of PS (AOS-1, NAOS-1) or PO (AO-1, NAO-1). Cleavage of the dye MTT to form an insoluble formazan product was measured as an indirect measure of the number of viable cells as described. The result is representative of three experiments.

FIG. 13

Phosphorothioate-modified CpG ODN are inactive in activation of NF-κB-dependent transcription. RAW264a4 cells, with an integrated HIV-1-LTR-luciferase reporter gene, were incubated for two hours with the ODN at the indicated concentrations prior to being harvested for determination of luciferase activity. The activity observed in unstimulated cells is arbitrarily given a value of 1. Results are the average of triplicates. The experiments are representative of at least three.

FIG. 14

Phosphorothioate-modified CpG ODN fail to down-modulate the CSF-1R from the surface of bone marrow-derived macrophages (BMM). BMM were starved of CSF-1 overnight then incubated with the indicated concentrations of ODN, fixed, and immunostained for surface expression of CSF-1R using the specific monoclonal antibody AFS-98. Panel A shows representative FACS profiles from one experiment. Panel B shows analysis of the proportion of cells showing down-modulated CSF-1R (based upon gating into the M1 fraction shown in Panel A) from two separate experiments where cells were incubated with the indicated concentrations of ODN.

FIG. 15

Phosphorothioate-modified ODN induce phosphorylation of ERK-1/2 more slowly than phosphodiesters. Bone marrow-derived macrophages were

starved of CSF-1 overnight, then incubated for the indicated times with 3 μM AO-1 or AOS-1 prior to lysis and Western blotting to detect ERK-1/2 phosphorylation as shown. A representative experiment is shown.

FIG. 16

Phosphorothioate-modified ODN prevent activation of NF-κB-dependent transcription by CpG DNA. RAW264a4 cells, with integrated HIV-1-LTR-luciferase reporter gene, were treated with AO-1 at 2 μM for 2hrs prior to harvesting and determination of luciferase. Unstimulated luciferase activity is given an arbitrary value of 1. In panel A, cells were either pretreated for 2hrs, or co-treated with an equimolar concentration of non-stimulatory PO-ODN (NAO-1) or PS-ODN (NAOS-1). In panel B, the concentration of AO-1 tested was 5μM, which is just maximal, and a 6 fold excess of NAO-1 was added.

FIG. 17

Inhibition of CpG DNA response by phosphorothioate-modified ODN does not require preincubation. RAW264a4 cells, with integrated HIV-1-LTR-luciferase reporter gene, were treated with 2 μM AO-1 for 2hrs prior to harvesting for measurement of luciferase activity. AOS-1 at equimolar concentration was added at the times indicated relative to the addition of AO-1. Results are the average of triplicates of a representative experiment.

FIG. 18

Phosphorothioate-modified ODN prevent activation of nitrite production induced by CpG DNA. RAW264 cells were primed with interferon-γ then treated for 24hrs with 1μM AO-1 or 5ng/ml LPS as indicated. NAO-1, or NAOS-1 were added at the concentrations shown, either alone, or 30 min prior to LPS or AO-1. The cells were incubated for a further 24 hr then nitrite production was determined. Results are the average of triplicates from a representative experiment.

FIG. 19

CCGG-containing ODN do not prevent activation by CpG DNA. RAW264 cells primed with interferon-γ were incubated with the indicated

concentrations of the PO-ODN IO-2, which has the same sequence as AO-1 except the core stimulatory motif is CCGG instead of ACGT. After 24 hr, cells were harvested for determination of nitrite production after 24 hr. Results are the average of triplicates.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is predicated, at least in part, on the present inventors discovery that RPA is the intracellular receptor for immunostimulatory DNA. This discovery led from the observations by the present inventors that assays which utilize macrophages, and macrophage-derived cell lines, can identify immunostimulatory DNA. Further to this, the present inventors have dissected the mechanisms underlying recognition of immunostimulatory DNA and have discerned differences between phosphodiester-backbone DNA and phosphorothioate-backbone DNA. This has enabled the present inventors to propose means by which immunostimulatory agonists and antagonists may be designed.

As used herein, "*DNA*" is a dexoxyribonucleic acid in single-or double-stranded form, inclusive of single- or double-stranded oligonucleotides and plasmids such as used in gene therapy or DNA vaccination. DNA normally comprises the purine bases adenine (A) and guanine (G), and the pyrimidine bases cytosine (C) and thymidine (T). However, the skilled person will also appreciate that DNA may include modified bases such as methylcytosine, inosine, methylinosine, methyladenosine, thiouridine and methylcytosine, for example.

As used herein, "*immunostimulatory DNA*" is DNA which elicits or potentiates an immune response by cells of the vertebrate immune system, preferably the mammalian immune system, and more preferably the human immune system. Suitably, responsive cells of the immune system include macrophages, NK cells, dendritic cells, B cells and T cells.

As used herein, a "*macrophage*" is a cell of the macrophage-monocyte myeloid lineage, preferably in mammals or more preferably in humans, or a cell line derived therefrom. This definition therefore includes

and encompasses primary macrophages such as bone marrow-derived macrophages (BMM) and cell lines such as MonoMac6, RAW264 and derivates thereof such as RAW264a4. A useful discussion of monocyte-macrophage development and function can be found, for example, in Chapter 11 of IMMUNOLOGY 3rd Ed. Roitt *et al.*(Mosby-Year Book Europe Ltd), which is herein incorporated by reference.

As used herein "*oligonucleotide*", abbreviated herein to "*ODN*", is single-stranded or double-stranded DNA comprising between six (6) and one hundred (100) contiguous nucleotides, or preferably fifteen (15) to thirty (30) nucleotides. An oligonucleotide may have naturally-occurring phosphodiester linkages between constituent bases, abbreviated herein to "*PO-ODN*", or phosphorothioate linkages, abbreviated herein to "*PS-ODN*". Phosphorothiaote linkages are created by replacing a non-bridging oxygen atom of the internucleotide phosphate group with a sulfur, to thereby create a thioester linkage. It will also be appreciated that oligonucleotides may be modified by addition of peptide linkages, enzymes, biotin, radiolabel, fluorochromes, lipids and carbohydrates.

For use *in vivo*, nucleic acids are preferably relatively resistant to degradation (e.g., are stabilized). A "*stabilized nucleic acid*" shall mean a nucleic acid that is relatively resistant to *in vivo* degradation (e.g. via an exo- or endo-nuclease). Stabilization can be a function of length or secondary structure. Unmethylated CpG oligonucleotides that are tens to hundreds of kbs long are relatively resistant to *in vivo* degradation. For shorter CpG oligonucleotides, secondary structure can stabilize and increase their effect. For example, if the 3' end of an oligonucleotide has self-complementarity to an upstream region, so that it can fold back and form a stem loop type of structure, then the oligonucleotide becomes stabilized and therefore exhibits more activity. Alternatively, nucleic acid stabilization can be accomplished via phosphate backbone modifications. Preferred stabilized oligonucleotides of the instant invention have a modified backbone. It has been demonstrated that modification of the oligonucleotide

backbone provides enhanced activity of the CpG oligonucleotides when administered *in vivo*. These stabilized structures are preferred because the CpG molecules of the invention have at least a partial modified backbone. CpG constructs, including at least two phosphorothioate linkages at the 5 end of the oligonucleotide and multiple phosphorothioate linkages at the 3' end, preferably 5', provide maximal activity and protect the oligonucleotide from degradation by intracellular exo- and endo-nucleases. Other modified oligonucleotides include phosphodiester modified oligonucleotides, combinations of phosphodiester and phosphorothioate oligonucleotide, methylphosphonate, methylphosphorothioate, phosphorodithioate, and combinations thereof. Each of these combinations and their particular effects on immune cells is discussed in more detail in PCT Published Patent Application Nos. PCT/US95/01570 and PCT/US97/19791 claiming priority from U.S. Serial Nos. 08/386,063 and 08/960,774, filed on February 7, 1995 and October 30, 1997 respectively, the entire contents of which are hereby incorporated by reference. It is believed that these modified oligonucleotides may show more stimulatory activity due to enhanced nuclease resistance, increased cellular uptake, increased protein binding, and/or altered intracellular localization.

Modified backbones such as phosphorothioates may be synthesized using automated techniques employing either phosphoramidate or H-phosphonate chemistries. Aryl-and alkyl-phosphonates can be made, e.g., as described in U.S. Patent No. 4,469,863; and alkylphosphotriesters (in which the charged oxygen moiety is alkylated as described in U.S. Patent No. 5,023,243 and European Patent No. 092,574) can be prepared by automated solid phase synthesis using commercially available reagents. Methods for making other DNA backbone modifications and substitutions have been described (Uhlmann & Peyman,1990, Chem. Rev. **90** 544; Goodchild, 1990, Bioconjugate Chem. **1** 1650).

Both phosphorothioate and phosphodiester oligonucleotides containing CpG motifs are active in immune cells. However, based on the

concentration needed to induce CpG specific effects, the nuclease resistant phosphorothioate backbone CpG oligonucleotides are more potent.

Other stabilized oligonucleotides include: nonionic DNA analogs, such as alkyl- and aryl-phosphates (in which the charged phosphonate oxygen is replaced by an alkyl or aryl group), phosphodiester and alkylphosphotriesters, in which the charged oxygen moiety is alkylated. Oligonucleotides which contain diol, such as tetraethyleneglycol or hexaethyleneglycol, at either or both termini have also been shown to be substantially resistant to nuclease degradation.

Oligonucleotides are readily available in synthetic form, and can be "made to order" from a variety of commercial and laboratory sources. Oligonucleotides generated by digestion of nucleic acids are also contemplated.

It will be appreciated that the method according to the first-mentioned aspect of the invention provides a means whereby the immunostimulatory capacity of DNA can be assessed. The first aspect of the invention makes particular use of the discovery that RPA is the receptor for immunostimulatory DNA, and allows direct identification of immunostimulatory DNA by virtue of its ability to bind isolated RPA.

In this regard, the present inventors have identified three RPA species hereinafter designated Band A and Band B and Band C respectively, which form complexes with immunostimulatory DNA. The proposed subunit composition of Bands A-C will be discussed in detail hereinafter.

By "*isolated*" is meant removed from a natural state, such as the cellular or tissue environment in which a biomolecule exists naturally, and includes recombinant synthetic biomolecules.

By "*RPA subunit*" is meant an intact RPA protein or fragment thereof which binds immunostimulatory DNA. For example, an RPA subunit includes 70kD protein or a 55kD fragment thereof, a 32-34 kD protein or a 14 kD protein which are capable of combining to form an RPA complex. As

will be described hereinafter, RPA subunits have been identified in a variety of animal species, each subunit encoded by a distinct gene.

Isolated RPA subunits may be purified recombinant, purified from a cellular source, or in the form of a nuclear or cytosolic extract. Recombinant RPA may be produced in bacteria, insect cells (e.g. by baculovirus expression in Sf9 cells) or in mammalian cells by techniques well known in the art, such as provided in Chapter 5 of CURRENT PROTOCOLS IN PROTEIN SCIENCE (Coligan *et al.*, Eds; John Wiley & Sons) which is herein incorporated by reference.

Recombinant polynucleotides which encode RPA subunits are readily available to the skilled person. In this regard, there are a number of mammalian RPA cDNAs, for example, the nucleotide sequences of which are available through the NCBI *Entrez* database. Of particular note are:-

(i) human RPA 70kD subunit (RPA70) cDNA; Accession number M63488; Erdile *et al.*, 1991, J. Biol. Chem. **266** 12090;

(ii) human RPA 32 kD subunit (RPA34) cDNA; Accession number J05249; Erdile *et al.*, 1990, J. Biol. Chem. **265** 3177; and

(iii) human RPA 14kD subunit (RPA14) cDNA; Accession number L07493; Umbricht *et al.*, 1993, J. Biol. Chem. **268** 6131;

each database submission and corresponding publication incorporated herein by reference.

Recombinant RPA protein subunits may be prepared as fusion proteins together with appropriate fusion partners such as glutathione-S-transferase, hexahistidine, and maltose-binding protein as are well known in the art. Indeed, kits are commercially available which facilitate construction of plasmids encoding recombinant fusion proteins and which facilitate fusion protein purification.

Formation of RPA-containing complexes, and detection

thereof, may be performed using an Electrophoretic Mobility Shift Assay (EMSA) or solid phase binding assays. Appropriate solid phase assays include immobilizing either RPA or said sample to a solid matrix such as microtitre plate, BIACORE sensor chip (as will be discussed in more detail hereinafter), magnetic bead (e.g. DynaBead or BioMag bead) or synthetic polymer (e.g. sepharose) beads, and detecting formation of said complex on said solid matrix.

Preferably, EMSA is used..

As used herein "*EMSA*" is a technique which detects formation of a complex between DNA and protein, for example RPA present in a macrophage-derived nuclear or cytosolic extract, and electrophoretic separation of the DNA-protein complexes from relatively lower molecular weight non-complexed DNA. Preferably, separation is by non-denaturing polyacrylamide gel electrophoresis (PAGE). Alternatively, agarose gel electrophoresis is used. Detection of RPA-containing complexes and non-complexed DNA is achieved by virtue of the DNA being associated with a label. Suitable labels include radionuclides or fluorescent labels such as TET, 6-FAM, ROX, Oregon Green or HEX. Preferably, the label is ^{32}P.

Preferably, EMSA includes a "*supershift*" step whereby RPA-containing complexes are formed by addition of an anti-RPA antibody or antibody fragment during formation of said DNA-protein complex. Such supershifted complexes have higher relative molecular weight than DNA-protein complexes in the absence of added antibody, and hence are readily discernible following size separation such as by PAGE. The particular advantage of this step is that the antibody provides a more specific means of identifying RPA-containing complexes.

Anti-RPA antibodies are well known and widely available in the art readily. Furthermore, anti-RPA antibodies are available from commercial sources such as NeoMarkers and Serotec.

A preferred EMSA method aplicable to RPA may be found, for example, in Ross *et al.*, 1994, Oncogene **9** 121 which is herein incorporated

by reference, and which forms the basis of a detailed description of an EMSA method applicable to the method of the invention as provided hereinafter.

In an embodiment, the isolated RPA is recombinant synthetic RPA produced according the methods such as already described. It is envisaged that different subunit compositions may be provided (e.g. comprising RPA70, RPA34, RPA14 and the aforementioned 55 kD proteolytic fragment of RPA70). The kit may be applicable to EMSA or solid phase binding assays as previously discussed. In this regard, additional components may be provided such as pre-cast polyacrylamide gels, molecular size standards or RPA immobilized to beads, microtitre plates or other solid matrices. Furthermore, control reagents may be included such as a known immunostimulatory DNA standard, and a non-immunostimulatory DNA "negative control".

With regard to aspects of the invention relating to macrophage-based methods of identifying immunostimulatory DNA, cell cycle arrest is a specific response of macrophage cells to immunostimulatory DNA. The particular stage at which cell cycle arrests is at the G_1 to S-phase transition. Preferably, cell cycle status is measured by flow cytometric analysis of DNA content. Preferably, propidium iodide (PI) is used to stain total cellular DNA for flow cytometric analysis. However, it will be appreciated that other nucleic acid stains can be used, including acridine orange, YO-PRO-1, Hoechst 33358, and ethidium bromide.

Alternatively, cell cycle status is measured by methods which employ bromodeoxyuridine (BrdU) incorporation and detection of cells which have entered S-phase using an anti-BrdU antibody. Detection may be performed either by flow cytometric analysis [where the anti-BrdU antibody is conjugated to a fluorochrome such as fluorescein isothiocyanate (FITC), Cy5, Cy3, tetramethyl rhodamine iosothicyanate (TRITC), Texas Red or phycoerythrin (PE), for example], or by histochemical analysis (where the anti-BrdU antibody is conjugated to alkaline phosphatase or horseradish

peroxidase, for example). It will also be apparent to one skilled in the art that flow cytometric analysis of DNA content can be combined with BrdU incorporation to provide a particularly sensitive bivariate analysis of cell cycle status. A detailed discussion of cell cycle analysis, DNA stains, fluorochromes, flow cytometry and methods of BrdU detection is provided in Chapter 7 of PRACTICAL FLOW CYTOMETRY (3rd ED) by Howard M Shapiro (John Wiley & Sons), which is herein incorporated by reference.

With regard to CSF-1R, this receptor (also known as c-*fms*) is constitutively expressed at the cell surface of primary macrophages (eg. BMMs) and a variety of monocyte-macrophage lineage cell lines such as RAW264 and monoMac6. Suitably, measurement of CSF-1R is performed using an anti-CSF-1R mAb. Preferably, the anti-CSF-1R antibody is mAb AFS-98 as described in Sudo *et al.*, 1995, Oncogene **11** 2469, which is herein incorporated by reference.

Preferably, the anti-CSF-1R antibody is detected by a secondary antibody conjugated to Cy5, or alternatively to another fluorochrome such as FITC, TRITC, PE or Texas Red. It is also envisaged that the anti-CSF-1R antibody may be directly conjugated to one of the abovementioned fluorochromes. Preferably, measurement of CSF-1R is performed by flow cytometric analysis of CSF-1R expression or by immunofluorescence microscopy, as will be described in detail hereinafter.

Other techniques for measuring cell surface CSF-1R expression include:-

(i) radioiodination of intact cells or membranes followed by immunoprecipitation (using anti-CSF-1R antibody); or

(ii) biotinylation of intact cells or membranes followed by affinity chromatography (using streptavidin-sepharose) and immunoblotting (using anti-CSF-1R antibody).

Strategies such as outlined in (i) and (ii) are well known in the art, and exemplary experimental protocols may be found in Chapter 3 of

CURRENT PROTOCOLS IN PROTEIN SCIENCE (Coligan *et al.*, Eds; John Wiley & Sons) which is herein incorporated by reference.

The present invention also contemplates immunostimulatory DNA agonists and antagonists. As used herein, an "*agonist*" causes immune system cells, preferably macrophage cells, to respond in a fashion similar to their response to immunostimulatory DNA. Conversely, an "*antagonist*" will inhibit, prevent, reduce or minimize a cellular response to immunostimulatory DNA.

In one embodiment, agonists and/or antagonists are identified by their ability to compete with immunostimulatory DNA, and hence prevent formation of a complex between RPA and immunostimulatory DNA. Detection of the presence or absence of said complex may be according to the method of the first-mentioned aspect of the invention.

In another embodiment, antagonists and/or agonists are identified which do not compete with immunostimulatory DNA, but are identified by binding RPA at a site other than that which binds immunostimulatory DNA. This embodiment is preferably performed using a BIACORE sensor chip with immobilized RPA to detect binding of an agonist or antagonist. BIACORE technology is increasingly well known in the art, and exemplary references applicable to this technology are provided by Malmqvist & Karlsson, 1997, Curr. Opin. Chem. Biol. **1** 378 and Fivash *et al.*, 1998, Curr Opin. Biotechnol. **9** 97, which are herein incorporated by reference.

However, it will also be understood that an antagonist cannot be distinguished from an agonist according to the aforementioned embodiments, but can subsequently be distinguished by a macrophage-based assay of the invention. Another useful macrophage-based assays applicable in this regard utilizes RAW264a4 cells, which have a HIV LTR-luciferase construct stably integrated into their genome (Stacey *et al.*, 1996, *supra*; Sweet *et al.*, 1998, *supra*). These cells respond to immunostimulatory DNA by expression of luciferase which can be readily measured in a semi-

automated fashion. Yet another useful assay is measurement of nitric oxide (NO) production by interferon-γ-primed macrophages in response to immunostimulatory DNA (Stacey *et al.*, 1996, *supra*; Sweet *et al.*, 1998, *supra* . Examples of the RAW264a4 luciferase assay and the NO assay will be provided in detail hereinafter.

In light of the foregoing, it is envisaged that the methods of the invention may be used to identify immunostimulatory DNA, and agonists or antagonists thereof, in directed or high-throughput screens. Potential agonists and antagonists could be known compounds of unknown efficacy with respect to immunostimulation, the products of combinatorial chemistry, or be derived from natural sources. Such agonists and antagonists could become candidate drugs which mimic or block the effects of immunostimulatory DNA.

As will be described in detail hereinafter, the phosphodiester backbone of PS-ODN appears to inhibit CSF-1R down-regulation by macrophages in response to immunostimulatory DNA, and is thereby an example of a class of antagonist.

Based on the known properties of immunostimulatory DNA, novel agonists or antagonists could be used as:-

(i) vaccine adjuvants;

(ii) immunotherapy for any form of cancer, allergic diseases (e.g. asthma);

(iii) immunotherapy for both chronic and acute inflammatory diseases and inflammatory autoimmune diseases (including but not restricted to systemic lupus erythematosus, arthritis, psoriasis, gingivitis, sarcoidosis, multiple sclerosis, colitis and ileitis); and

(iv) activators of innate immune responses to protect against infection or to ameliorate symptoms of infection (e.g. septic shock).

Small molecule agonists or antagonists of immunostimulatory

DNA may possess the particular advantage of displaying improved absorption by the oral route, compared to CpG-containing DNA for example, and be far more economical to produce on a commercial scale.

Therefore, the present invention contemplates therapeutic and prophylactic compositions comprising immunostimulatory DNA, agonists and antagonists thereof, together with an acceptable carrier or delivery agent. Compositions for delivery of immunostimulatory DNA, or agonists and antagonists in the form of DNA, include lyophilized dry powder formulations which comprise lipids such as phosphatidyl ethanolamine, phosphatidyl choline, cationic bile salts and a variety of ethylphosphocholine- and cholestyl ester-containing derivatives, such as provided in U.S. Patent No. 5,811,406, which is herein incorporated by reference. Also contemplated are compositions in the form of DNA-hypericin polyamine complexes, such as described in U.S. Patent No. 5,824,654, which is herein incorporated by reference.

In light of the foregoing, the skilled person is referred to Mahon *et al.*, 1998, Crit. Rev. Biotechnol. **18** 257 for a recent review of new approaches to delivery of DNA for therapeutic purposes, which is herein incorporated by reference.

Other carriers, diluents and delivery agents generally applicable to therapeutic or prophylactic compositions are also contemplated. These generally include a solid or liquid filler, diluent or encapsulating substance which may be safely used in systemic administration. Depending upon the particular route of administration, a variety of pharmaceutically-acceptable carriers or diluents, well known in the art may be used. These may be selected from a group including sugars, starches, cellulose and its derivatives, malt, gelatine, talc, calcium sulfate, vegetable oils, synthetic oils, polyols, alginic acid, phosphate buffered solutions, emulsifiers, isotonic saline, and pyrogen-free water.

Dosage forms include tablets, dispersions, suspensions, injections, solutions, syrups, troches, capsules, suppositories, topically

administered powders, aerosols and emulsions, transdermal patches and the like. These dosage forms may also include controlled release devices or other forms of implants modified to act in this fashion. Controlled release of the therapeutic agent may be effected by coating the same, for example, with hydrophobic polymers including acrylic resins, waxes, higher aliphatic alcohols, polylactic and polyglycolic acids and certain cellulose derivatives such as hydroxypropylmethyl cellulose. In addition, the controlled release may be effected by using other polymer matrices, liposomes and/or microspheres.

Compositions of the present invention may be suitable for administration orally or by injection, and in such cases may be presented as discrete units such as capsules, sachets or tablets, as a powder or granules, or as a solution or a suspension in an aqueous liquid, a non-aqueous liquid, an oil-in-water emulsion or a water-in-oil liquid emulsion.

So that the present invention can be understood in more detail, the skilled person is directed to the following non-limiting experimental examples.

EXAMPLES

EXAMPLE 1

General Materials and Methods

1.1 *Cells*

RAW264 cells were obtained from the American Type Culture Collection (ATCC). To avoid phenotypic drift during prolonged culture, the original aliquot obtained from ATCC was expanded without subculture and frozen. Cells were maintained in culture for no more than one month before reinitiating culture from a fresh vial. Bone marrow-derived macrophages (BMM) were obtained by cultivation of femoral bone marrow cells for one week on bacteriological petri dishes in recombinant human macrophage colony-stimulating factor (CSF-1; a gift from Chiron Corp, Emeryville, CA). Adherent cells were harvested by squirting them from the plastic surface with medium using a 20 mL syringe and 18 gauge needle. The medium in

all experiments was RPMI-1640 plus 10% heat-inactivated fetal bovine serum with 20U/ml penicillin and 20μg/ml streptomycin. Serum was screened for low LPS contamination using RAW264a4 cells. RAW264a4 is a stable transfectant of RAW264 with an integrated HIV-1-LTR-luciferase reporter gene that is activated by low concentrations of LPS, or CpG DNA.

1.2 *Luciferase reporter assays*

The RAW264a4 macrophage cell line, a stable transfectant of RAW264 with integrated HIV-1-LTR driving a luciferase reporter gene, was described previously (Sweet *et al.*, 1998, *supra*). Briefly, 2 x 10^5 cells were cultivated overnight in 24 well plates in 1 ml of medium overnight, then treated for the desired time with agonist, washed and extracted for luciferase activity determination using the Luciferase Reporter Gene kit from Boehringer-Mannheim and the 1450 Microbeta TRILUX plate luminometer.

1.3 *Nitrite Assay*

The assay of nitrite using the Griess reagent was used as an indicator of NO production by both BMM and the murine macrophage-like cell line RAW-264 as described previously. Briefly, 4 x 10^4 RAW-264 or 10^5 BMM (with 1000 U/ml CSF-1) were plated in 96-well plates in 200 µl supplemented RPMI and cultured overnight. The following day cells were primed with 20 U/ml recombinant murine interferon-γ (R&D systems, Minneapolis, MI) for 2 hrs, followed by addition of the triggering stimulus. After 24 hrs supernatants were removed and assayed for nitrite.

1.4 *Viable cells*

The reduction of the dye MTT (Sigma; 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide) was used as a indirect assay of the number of viable cells. BMMs were harvested and plated in CSF-1-free medium for 18hrs (4x 10^4 cells/well in 0.2ml in a 96 well plate). The desired additions were made, and the cells were incubated for a further 48 hrs, prior to assessment of MTT reduction.

1.5 *Oligonucleotides and LPS*

Phosphodiester (PO) and phosphorothioate (PS)

oligonucleotides (ODN) were purchased from Geneworks (Adelaide, Australia) or Pacific Oligos (Brisbane, Australia). Oligonucleotide sequences were:-

(i) AO-1 (SEQ ID NO:1): 5'-GCT CAT GAC GTT CCT GAT GCT G-3';

(ii) AOM-1 (SEQ ID NO:2) is a version of AO-1 where the C of ACGT is methylated: 5'-GCT CAT GAC GTT CCT GAT GCT G-3';

(iii) NAO-1 (SEQ ID NO:3) is the same as AO-1, except that the core CG is transverted to GC: 5' GCT CAT GAG CTT CCT GAT GCT G 3';

(iv) AOS-1 (SEQ ID NO:4) and NAOS-1 (SEQ ID NO:5) are the phosphorothioates corresponding to (i) and (iii) respectively;

(v) IO-2 (SEQ ID NO:6) has the same sequence as AO-1 except that the core ACGT motif is replaced by CCGG: 5'-GCT CAT GCC GGT CCT GAT GCT G-3';

(vi) AAC-22 (SEQ ID NO:7):5'-ACC GAT AAC GTT GCC GGT GAC G-3';

(vii) ACC-22 (SEQ ID NO:8): 5'- ACC GAT ACC GGT GCC GGT GAC G-3';

(viii) M12 (SEQ ID NO:9): 5'-ATC CAT AAC GTT CCA GAA GCT G-3';

(ix) PU-1 (SEQ ID NO:10): 5'- GTA GGA CCG GAA GTG GGA GT-3';

(x) Oct (SEQ ID NO: 11): 5'- AAT TCG AGC TCG GTA CCC GAT CCT AGC CCC TCT ATG CAA ATG AGA AGC ATT CCT TTC GAA TTG GGG ATC CTC TAG AGT CGA CCT GCA GGC ATG CAA GCT-3';

(xi) 1668 (SEQ ID NO:12): 5'-TCC ATG ACG TTC CTG ATG CT- 3';

(xii) IL12-p40 (SEQ ID NO:13): 5'- GCT ATG ACG TTC CAA GGG-3'; and

(xiii) SAAC (SEQ ID NO:14) has the same sequence as AAC-22, but with a phosphorothioate backbone.

LPS from *salmonella minnesota* Re595 was purchased from Sigma and was dissolved as a 10mg/ml stock solution in 0.1% triethylamine and sonicated.

1.6 *Cell proliferation/cell cycle and apoptosis measurements*.

Bone marrow-derived macrophages (BMM) were prepared by cultivation of CD1 outbred mouse femoral bone marrow cells in recombinant CSF-1 and harvested after 6-8 days as previously described (Stacey *et al.*, 1995, Mol. Cell Biol. **15** 3430, which is herein incorporated by reference). For cell proliferation assays, cells were cultivated in medium for the desired time with or without CSF-1 and other additions, then a measure of the number of viable cells was obtained by incubation in the vital dye MTT as detailed previously (Stacey *et al.*, 1993, Immunol. Cell Biol. **71** 75). To assess cell cycle status, 10^7 cells preincubated with the desired stimuli were harvested, fixed and permeabilised by resuspension in 1 ml of PBS followed by addition of 3 ml of 95% ethanol and storage for at least 1 hr. Cells were washed in PBS, harvested and then incubated for 30 min in the dark with 2 ml of PBS containing RNaseA (0.2 mg/ml) and propidium iodide (40 µg/ml PI). Stained cells were analysed to determine the proportions in G_1, S, G_2 and M stages of the cell cycle using a Becton-Dickinson FACSCalibur flow cytometer. The proportion of apoptotic cells in similar incubations was assessed on the basis of exclusion of the vital dye propidium iodide (PI). Cells were incubated in 40 µg/ml PI in complete medium for 1.5 hrs at 37°C then analysed on a FACSCalibur flow cytometer.

1.7 *Immunofluorescence*

For direct visualisation, BMM were harvested, cultivated overnight on coverslips with the desired stimulus (e.g. with or without CSF-1) then treated or not with the appropriate stimulus and fixed with 4%

paraformaldehyde in phosphate buffered saline. Fixed cells were permeabilised with 0.1% Triton X-100 in PBS for 5 min, washed 3 times with PBS, then 0.5% BSA in PBS for 15 min. Fixed and permeabilized cells were incubated with anti-CSF-1R monoclonal antibody (obtained from Dr J. Hamilton, University of Melbourne), for 90 mins, then Cy5-labelled $F(ab)_2$-goat anti-rat IgG second antibody (obtained from Serotec) with extensive washing in phosphate buffered saline (PBS) between each step.

For FACS analysis, BMM were harvested at the desired time, fixed for 5 min in 4% formaldehyde in PBS, then incubated in the same succession of reagents as for direct visualisation. Staining was assessed using a FACSCalibur flow cytometer.

To measure down-modulation of CSF-1R, BMM were starved of CSF-1 overnight then incubated with the desired treatments on bacteriological petri dishes, prior to being fixed and immunostained for surface expression of CSF-1R using the specific monoclonal antibody AFS98 (Sudo *et al.*, 1995, *supra*) as described previously herein.

1.8 *Phosphorylation of MAPKinases*

Cell extracts and Western blotting for the determination of the phosphorylation state of the MAPKinases ERK-1 and ERK-2 were carried out as described previously (Sweet & Hume, 1995, J. Inflamm. **45** 126, which is herein incorporated by reference).

1.9 *Electrophoretic mobility shift assays*

Approximately 10^7 cells were lysed by hypotonic swelling followed by incubation in 0.1% NP40 detergent as described previously (Ross *et al.*, 1994, *supra*). Nuclei were separated from cytoplasm by centrifugation and the cytoplasmic extract was made up to a volume of 50 µl. Nuclear proteins were extracted from nuclei and made up to 50 µl in extraction buffer. Single-stranded or double-stranded oligonucleotide (DNA) probes were end-labelled with ^{32}P, and incubated with 2 µg of cytoplasmic or nuclear protein extract for 30 minutes prior to separation on non-denaturing 7.5% polyacrylamide gel electrophoresis (PAGE). For supershift

studies, monoclonal antibodies (mAb) against human RPA subunits RPA70 and RPA34 were provided by Dr. Bruce Stillman of Cold Spring Harbor Laboratory, NY USA, and were used directly as hybridoma supernatants. A rabbit heteroantiserum raised against purified human RPA (all three subunits), was a gift from Dr. Guiseppe Baldacci (Institut de Recherches sur Le Cancer, Villejuif, France). Irrelevant antiserum or hybridoma supernatant was added to control incubations.

For UV cross-linking experiments, EMSA gels were exposed directly to UV transillumination and the positions of bands were identified by exposure of the wet gel to film. Bands were excised, extracted, and subjected to further separation by SDS-PAGE as described previously (Ross *et al.*, 1994, *supra*)

EXAMPLE 2

Macrophage-based detection of immunostimulatory DNA

2.1 *Determination of the optimal DNA structure recognised by murine macrophages*

In earlier studies the present inventors described a cell line assay for LPS in which the HIV-1-LTR driving luciferase enzyme expression is stably integrated into the DNA of the murine macrophage cell line RAW264 (Sweet & Hume, 1995, *supra*). Addition of LPS to these cells (RAW264a4 cells) caused rapid activation of luciferase expression, peaking after around 2 hrs. This cell line also responds to plasmid DNA or activating oligonucleotides in a similar fashion. (Stacey *et al.*, 1996, *supra*; Sweet *et al.*, 1998, *supra*). Others (Lipford *et al.*, 1997, Eur. J. Immunol. **27** 3420) have claimed that different specific CpG DNA sequences can induce IL-12, but not TNF-α, in macrophages, with the implication that such "selective" oligonucleotides could harness therapeutic activities of CpG whilst avoiding toxic activities associated with induction of genes such as TNF-α. Most such studies have been based upon the use of phosphorothioate-modified oligonucleotides. Whilst of therapeutic relevance, such modification adds another variable since the phosphorothioate backbone is biologically active

and may be toxic at high concentrations. The present inventors therefore sought to ascertain whether differences in apparent efficacy might relate to shifts in dose response curve that are not evident when a single concentration is examined. Such studies are expedited by the availability of an economical, rapid and convenient bioassay such as provided by RAW264a4 cells. FIG. 1A compares the relative activities of a range of published phosphodiester oligonucleotides with varying activities on the induction of luciferase activity in a4 cells. Examined at a single concentration, the AO-1 oligonucleotide is the most active phosphodiester oligonucleotide tested in macrophage assays, and is more active than the phosphodiester equivalent of the 1668 oligonucleotide (Yi *et al.*, 1996, J. Immunol. **157** 4918) and the IL12-p40 phosphorothioate ODN (described by Lipford *et al.*, 1997, *supra* as a weak activator that did not induce TNF-α). The difference between AO-1 and 1668 is simply an increase in length by adding terminal G nucleotides and inversion of the two most 5' bases in ODN1668. ODN1668 and IL12p40 have the same core 10 bp sequence and differ in flanking bases on either side. Mutation of either one of the single flanking bases reduced the activity of the 1668 ODN. FIG. 1B shows that the difference in activity between ODN 1668 and IL12p40 is greater than is evident from a single concentration comparison, and results primarily from a change in dose response curve (i.e. a 5 fold shift in half-maximal concentration). Mutation in 1668 ODN of either of the flanking bases that distinguishes the two ODNs reduced the activity towards that of the less active IL12p40 ODN. The differences in efficacy of different ODNs are time independent; like the response to LPS, the response to each of the oligonucleotides reaches a peak after 2-3 hrs (FIG. 1C).

Activation of the HIV-1-LTR in the cell line assay in general parallels activation of TNF-α mRNA. Lipford *et al.* 1997, *supra* reported that the IL-12p40 ODN did not induce TNF-α mRNA or protein secretion, but the observation was made only at a single concentration (1 μM) and, as noted above, the ODN was a phosphorothioate derivative.

FIG. 2 compares the dose response curves for induction of TNF-α protein release from RAW264 macrophages. The conclusion is the same as for RAW264a4 cells. The difference in apparent efficacy of the different oligonucleotides simply reflects a shift in half-maximal activating concentration on the dose response curve. This conclusion is important in terms of defining the mechanism of CpG DNA action, since it obviates any necessity to propose more than one recognition molecule or signalling pathway.

In summary, the results in the RAW264a4 cell assay, which measures NF-κB-dependent transcriptional activation, indicate that the sequence specificity of the response to ODNs is comparatively loose. The motif that is recognised may extend over up to 16 nucleotides. Different oligonucleotides have different dose response curves in the assay, and because the curves are very steep, assays at a single concentration can give a misleading impression as to the absolute requirement for sequence specificity.

2.2 *Cell cycle status, proliferation and apoptosis by bacterial DNA*

Induction of NF-κB-dependent transcription is one hallmark of the response of mammalian cells to DNA damage induced by a range of genotoxic agents (Liu *et al.*, 1996, Nature **384** 273). Indeed, activation of the HIV-1-LTR has also been used as an indicator of such damage in fibroblasts (Miller *et al.*, 1997, Exp. Cell Res. **230** 9) and defective induction of NF-κB is associated with hypersensitivity to DNA damage-induced apoptosis (Jung *et al.*, 1998, Radiat. Res. **149** 596). It was therefore considered whether the response to immunostimulatory DNA by macrophages might include changes in cell cycle progression. One of the hallmarks of the response to damaged DNA (e.g. caused by ionizing irradiation) is cell cycle arrest, or depending upon the extent of the insult, inhibition of apoptosis (see Liu *et al.*, 1998, *supra*). Others have shown that CpG DNA can prevent apoptosis induced in a B lymphoma line induced by anti-IgM (MacFarlane *et al.*, 1997, *supra*; Yi *et al.*, 1996, *supra*; Yi *et al.*,

1998, *supra*), but interpretation of these results is complicated by the fact that CpG DNA is itself a mitogen for B cells. The present inventors therefore examined whether treatment with immunostimulatory DNA affects cell cycle progression in murine bone marrow-derived macrophages which depend upon the macrophage-specific growth factor CSF-1, for survival and proliferation. BMMs starved of CSF-1 analysed following a propidium iodide based vital staining procedure showed decreased dye exclusion indicative of reduced cell membrane integrity. Bacterial plasmid DNA or immunostimulatory ODN prevented apoptosis completely, to the same extent as readdition of CSF-1 or LPS (FIG. 3). In contrast to CSF-1, neither plasmid DNA or ODN allowed cells to progress into the S and G2M phases of the cell cycle. In fact, like LPS (Hume *et al.*, Lymphokine Res. **6** 127) CpG-containing DNA also caused almost complete inhibition of cell proliferation. This was evident by the reduction in the increase in cell number using a simple colorimetric assay for viable cells (data not shown). Cell cycle analysis using the FACS indicated that like LPS, CpG DNA caused a block to progression into S phase of the cell cycle (FIG. 4). These results indicate that both LPS and immunostimulatory DNA affect macrophages in a fashion similar to DNA damage in other cell types.

2.3 *CSF-1 receptor regulation in response to LPS and CpG DNA*

LPS has been shown previously to cause rapid down-regulation of CSF-1 binding to murine macrophages (Hume *et al.*, 1987, *supra*; Guilbert & Stanley, 1984, J. Immunol. Meth. **73** 17; Baccarini *et al.*, 1992, J. Immunol. **149** 2656). It was postulated by the present inventors that this response may underlie both the growth inhibition and survival described in the previous section. For this reason, the present inventors examined whether immunostimulatory DNA elicits CSF-1R down-regulation. FIG. 5A examines the expression of CSF-1 receptor on the surface of BMMs determined by immunofluorescence using an anti-CSF-1RmAb (Sudo *et al.*, 1995, Oncogene **11** 2469). The specificity of the staining is evident from the rapid down-regulation that occurs in response to CSF-1). Both LPS and

plasmid DNA or stimulatory ODN caused rapid disappearance of the CSF-1 receptor from the cell surface and its appearance in small cytoplasmic vesicles. These pictures also show that both treatments cause rapid spreading that is morphologically distinct from that elicited by CSF-1. At higher magnification it is evident that both treatments apparently deplete the intracellular pool of CSF-1R that can be distinguished in the presumptive perinuclear Golgi regions and cause the appearance of extensive cytoplasmic vesiculation, which manifests as granularity under phase contrast.

The time course of CSF-1R down modulation can be measured more readily by FACS using anti-CSF-1R mAb, which also gives single cell information. FIG. 5B compares down modulation by CSF-1, LPS and various DNAs after 1 hr. While immunostimulatory *E. coli* DNA caused down-modulation of CSF-1R, non-stimulatory DNA such as CTDNA and SSDNA did not. Stimulatory DNAs inclusive of *E. coli* DNA and active CpG-containing ODN caused down-modulation of CSF-1 receptor in an all-or-nothing manner as represented by bimodal surface expression profiles as shown in FIG. 5C & D.An ODN containing an inversion of the active CpG to GpC did not affect surface CSF-!R levels (FIG. 5D).

EXAMPLE 3

Identification of immunostimulatory DNA-binding proteins in macrophages

Based upon the macrophage cell-based assays, the recognition molecule that mediates responses to immunostimulatory DNA should distinguish bacterial dsDNA from methylated or mammalian DNA. It need not be absolutely sequence specific for CpG motifs in single-stranded DNA but might be expected to follow the hierarchy of stimulatory potential identified in the bioassays described above.

One previous study has reported that all detectable single-stranded DNA binding activity in the cytoplasm of human or rodent cells extracted with mild detergent lysis is immunoreactive with antibodies

directed against 70kD and/or 34kD subunits of RPA (Seroussi & Lavi, 1993, J. Biol. Chem. **268** 7147). FIG. 6A shows electrophoretic mobility shift assays (EMSA) using cytoplasmic and nuclear extracts produced from RAW264 cells together with AO-1 ODN. For comparison, the same extracts are probed with dsDNA probes having consensus sequences recognized by the macrophage-specific transcription factor PU.1, and the octamer transcription factors Oct-1 and Oct-2, both of which are expressed in macrophages (FIG. 6B). In each case, a low level of poly-dIC is present to reduce binding by a non-specific low affinity DNA binding protein that has been detected previously in mouse cells under similar conditions (Ross *et al.*, 1994, *supra*). The results show a complex set of bands that bind a single-stranded immunostimulatory ODN. In contrast to PU.1, and in even greater contrast to the octamer binding proteins (which are completely nuclear restricted), the observed set of bands are not specifically enriched in the nuclear extracts, nor are they specifically excluded. In general, the gel system used herein resolves three major bands of binding activity in cytoplasmic extracts, referred to as Bands A, B and C in order of increasing electrophoretic mobility. A 4th single-stranded DNA binding band (B') is evident solely in nuclear extracts in this experiment, but was occasionally observed in cytoplasmic extracts.

FIG. 6B shows that the major band present in EMSA analysis of RAW264 extracts (A + B) are also present when extract from the fibroblast cell line NIH3T3 is used.

The use of poly-dIC as a cold competitor was considered undesirable since it is also able to activate macrophages (Stacey *et al.*, 1996, *supra*). The present inventors therefore used sheared salmon-sperm DNA as a competitor. FIG. 6C shows a cold competition experiment in which increasing amounts of the DNA are added. In the extract shown there are 4 bands. Addition of competitor reduced binding of all of the bands, with some selectivity for the B' nuclear band discussed above. FIG. 6D shows a cold-competition experiment in which the complex formed with activating

ODN is cold-competed with plasmid DNA, which is immunostimulatory. The faster migrating bands (including bands B and C) were effectively cold competed with plasmid DNA.

The present inventors next considered whether the major binding activities could distinguish between activating and non-activating ODNs. FIG. 7 contains a series of comparisons of non-activating and activating ODNs. In FIG. 7A, the binding of ssDNA and dsDNA oligonucleotides is compared, in this case using the active ODN referred to previously as AAC-22 (Stacey *et al.*, 1996, *supra*). A series of independent extracts from RAW264 cells provided very similar patterns of binding, and despite the presence of the salmon sperm DNA, single-stranded and double-stranded activating ODN behaved similarly. Because probe is present in excess, it is not possible to determine with assurance whether this binding involves recognition of non-annealed ssDNA. Cold competition with an excess of either ssAAC-22, or the ACC ODN which lacks immunostimulatory activity, provides evidence that the activity does bind ssDNA preferentially. Furthermore, where ssAAC-22 could completely cold-compete all the bands, the inactive ODN was unable to do so at a similar concentration, indicating that both ssDNA and dsDNA binding is sequence specific.

To extend these findings, the present inventors performed dose-dependent cold-competition experiments. Panel 7B provides evidence for greater relative binding activity by the activating oligonucleotide (AAC-22) under the conditions of the assay. Panel 7C shows an independent comparison of relative binding of a different set of ODNs with unrelated flanking sequences to AAC-22; the strongly activating AO-1, a non-activating GC transversion (NAO-1) and an oligonucleotide in which the core CpG motif is methylated. In each case the same set of complexes was formed with the labelled oligonucleotide. AO-1 was the most effective cold competitor regardless of whether it was competing with self, or with the less active ODNs. Panel 7D confirms the findings in 7C with a comparative dose

response curve for cold competition by each of the ODNs for binding of AO-1, indicating that 5-fold higher amounts of NAO-1 or methylated ODN are required to compete to the same extent as AO-1. Finally, FIG. 7E shows a comparative cold competition for a strong (ODN1668) and weak (IL12p40) oligonucleotide that differ approximately 5-fold in relative activity in the RAW264a4 macrophage cell line assay (see above). Again, there was a 5-fold difference in the ability of the two ODNs to cold compete for binding to AO-1.

The affinity of RPA for ODN can be so high as to be effectively irreversible under the conditions of EMSA (Wold, 1997, *supra*). To determine whether this was the case for the cytoplasmic proteins in the present study, we examined the effectiveness of cold competitor added 15 minutes after the radiolabel (FIG. 8A). In this case, no release of labelled probe was observed even after a further 15 minutes of incubation for the slowest migrating band (Band A) whereas the faster migrating band B (C was not evident in this experiment) released the probe.

The binding activity of RPA is known to be sensitive to alterations in ionic composition of the binding buffer, particularly the addition of magnesium (Wold, 1997, *supra*). The present inventors therefore examined whether addition of Mg^{2+} or increased levels of K^+ to the EMSA binding reaction altered complex formation. FIG. 8B shows that monovalent cations increased the absolute binding activity of the abundant slow migrating complex. Conversely, Mg^{2+} above 2 mM caused a very substantial reduction in binding activity of the more slowly-migrating complex. These patterns are essentially consistent with binding activities of known forms of RPA (see discussion). Note that reduction/increase in binding activity of one of the three bands is not correlated with any change in other bands, suggesting that the alterations do not involve interconversion between different forms of DNA binding protein, but rather independent optimisation of the binding of each form.

To estimate the relative molecular weight of the ssDNA binding

proteins, we performed a UV cross linking on RAW264 cell extracts. Results are shown in FIG. 9. The faster migrating band (band C) produced a single complex of around 55 kD. The slowest migrating band (Band A, which binds irreversibly) was not sufficiently resolved from the next band (band B) to give a clearly distinctive signal in preliminary experiments. Band B produced a doublet of around 70-75 kD with a very weak signal co-migrating with the 55 kD band produced from band C. When the total binding activity of the cytoplasmic extract was examined, it resolved into three major bands, the 55 kD band, the 70-75 kD doublet seen with band B and an additional major band of 30-35 kD. The 70-75 kD and 30-35 kD bands are consistent with identity as RPA1 and RPA2 subunits respectively. The 55 kD complex is consistent with a known truncated product of RPA70 (Wold, 1997, *supra*; Seroussi & Lavi, 1993, *supra*). Indeed, it was found that limited digestion of the extracts with trypsin led to the disappearance of the slower migrating band A and appearance of a band that co-migrated with band C (not shown). Final confirmation of the identity of the three bands as forms of RPA was obtained using specific antisera. Because these antisera were raised against human proteins, we prepared an extract from the human macrophage line monoMac6 for comparison. Monoclonal antibody directed against human RPA70 or RPA34 completely supershifted the band B complex obtained from monoMac6 cells. Anti-RPA70 was inactive in mouse, but anti-RPA34 also shifted the faster migrating bands B&C. A heteroantiserum raised against purified human RPA also supershifted all the faster migrating bands (B&C) in both human and mouse (not shown). The specificity of these supershifts was confirmed by the absence of comparable changes in mobility with irrelevant monoclonal antibody or heteroantiserum at the same concentration.

EXAMPLE 5

Identification of inhibitory effects of PS-backbone

5.1 *PS-ODN can be more potent than PO-ODN in assays of CpG DNA activity in macrophages.*

RAW264 macrophages and primary bone marrow-derived acrophages (BMM) primed with interferon-γ respond to exposure to CpG DNA with expression of inducible nitric oxide synthetase mRNA and increased nitric oxide (NO) production (Stacey *et al.*, 1996, *supra*; Sweet *et al.*, 1998, *supra*). This assay provides a convenient and sensitive measure of the efficacy of immunostimulatory DNA in macrophages. PO-ODN and PS-ODN of the same sequence AO-1 and AOS-1, were compared for their ability to induce NO production in interferon-primed BMM and RAW-264 cells in FIGS. 11A and 11B respectively. The dose response profiles for the primary cells and the cell line are almost indistinguishable. Both ODNs were active. The PS-ODN always gave a lower maximum, but the major effect of the PS modification was on the half-maximal effective dose. Maximal response to AOS-1 was achieved at approximately 100-fold lower concentration than for AO-1 and further elevation of the concentration caused NO production to decline from the maximum. CpG specificity was preserved for the response to AOS-1, although low levels of NO production were observed occasionally with the PS-ODN NAOS-1, which differs only in having a reversal of the CpG motif to GpC. The same differential sensitivity of sequence identical PS-ODN and PO-ODN was observed with unrelated ODNs, including the PO-ODN AAC-22 utilised in previous studies (Stacey *et al.*, 1996, *supra*; Sweet *et al.*, 1998, *supra*) and the corresponding phosphorothioate SAAC-22. In both cases, the dose response curves to PS-ODNs displayed a clear maximum, above which higher concentrations of PS-ODN reduced NO production (data not shown). By contrast to LPS, which induces NO production without an absolute requirement for interferon-priming, even the active PS-ODN did not induce NO production when added as the sole stimulus (not shown).

Because the induction of NO production depends upon interferon-γ priming, the present inventors considered the possibility that interferon sensitised specifically to PS-ODN. The present inventors have shown elsewhere herein that CpG DNA can prevent apoptosis that occurs

in BMM deprived of the growth and survival factor, macrophage colony-stimulating factor (CSF-1). FIG. 12 compares the dose response curves for the anti-apoptotic activity of AO-1 and AOS-1. The assay used is based upon an indirect assessment of viable cells based upon reduction of the dye MTT. It has been validated in terms of counts of cell numbers and evidence of DNA fragmentation shown herein. In this assay AO-1 and AOS-1 both maintained cell viability in the absence of CSF-1, whereas the corresponding GpC tranversions lacked activity. In comparative dose-response curves AOS-1 was again approximately 100-fold more active than AO-1 (FIG. 12). The dose response curves for AOS-1 and AO-1 were similar in the cell viability and NO assays, suggesting that interferon-γ does not sensitise preferentially to phosphorothioate CpG DNA.

5.2 *PS-ODN are less active than PO-ODN in CpG activation of transcription and CSF-1R down-modulation*

Both the iNOS assay, and the anti-apoptotic assay, involve incubation for extended periods in culture where the relative stability of PS-ODN could be a determinant of outcome. Mechanistically, NF-κB was the first signalling molecule demonstrated to be activated by CpG DNA (Stacey *et al.*, 1996, *supra*). NF-κB-dependent transcription in macrophages has been assessed using an RAW-264a4 cell line. Both bacterial DNA, and stimulatory ODN (AO-1) were very active in this assay. By contrast, the PS-ODN AOS-1 was much less active than the corresponding PO-ODN AO-1 (FIG. 13A). The same pattern was observed with the PO-ODN AAC-22 and its corresponding PS-ODN SAAC-22 (FIG. 13B). Both ODNs caused a minimal activation of HIV-1-LTR at low concentration, but had little additional impact with increasing concentration.

The anti-apoptotic effect of CpG DNA in BMMs is preceded by acute and rapid down-modulation of the CSF-1 receptor from the cell surface, a response that occurs in an all-or-nothing manner at the single cell level. This assay permits an assessment of whether the low response of macrophages to PS-ODN reflects a partial response in individual cells. Dose

response analysis of CSF-1 receptor down-modulation was conducted with AOS-1 and AO-1 (FIGS. 14A and 14B). FIG. 14A shows the representative FACS profiles. Note that increasing AO-1 concentration increases the proportion of cells with completely down-modulated receptors (falling within the M1 gates). Similar to the observations made with activation of the HIV-1-LTR, PS-ODN were much less active than their PO-ODN counterparts. Whereas 95% of cells fully-down-modulated their CSF-1R within one hour with 3 μM AO-1, only 25% of cells had responded to 3 μM AOS-1 within the same time frame. The same trend was observed in a comparison of SAAC-22 and AAC-22 (FIG. 14C). FIG. 14C also reveals that the less active ODN, AAC-22, differs from AO-1 by approximately 5-fold in the concentration required to activate 50% of cells to down-modulate their CSF-1 receptors.

Maintenance of cell viability in CSF-1-starved BMMs has previously been associated with the ability of both LPS and CpG DNA to activate the MAP Kinases, ERK-1 and ERK-2 (Sweet & Hume, 1995, J. Inflamm. **45** 126, which is herein incorporated by reference). LPS causes very rapid activation of these enzymes, which can be detected by Western blotting using anti-phospho-ERK1/2 antibodies. A stimulatory ODN such as AO-1 acts more slowly than LPS, perhaps reflecting the time required for internalisation. FIG. 15 compares the time course of activation of ERK-1/2 in BMM by AO-1 and AOS-1. AO-1 caused maximal activation of ERK-1/2 phosphorylation after 20 minutes, followed by a small decline to stable, elevated steady state after 40 minutes. By contrast, AOS-1 had no significant effect after 20 minutes, and reached a maximum after 1 hr that was still less than the peak observed with AO-1.

5.3 *Differential Blockade of CpG DNA responses by non-CpG PS-ODN and PO-ODN*

Hacker *et al.*, 1998, EMBO J. **17** 6230 have presented evidence that endosomal uptake of PS-ODN can be saturated, and that non-CpG PS-ODNs can compete and specifically block the response to CpG ODNs. The question of whether the actions of CpG PO-ODN might be

similarly compromised by non-CpG ODN has not previously been addressed. Pre-incubation or co-treatment with an equimolar concentration of PO-ODN NAO-1 had no significant effect on activation of HIV-LTR-dependent luciferase activity in RAW264a4 cells induced by AO-1, whereas both pre- and co-treatment with an equimolar concentration of the equivalent PS-ODN NAOS-1 substantially diminished activation by AO-1 (FIG. 16A). Even a six-fold molar excess of the non-CpG PO-ODN NAO-1 had no significant effect on the response to AO-1 at a concentration that was near saturating in biological "CpG" activity (FIG. 16B). The effect of non-CpG PS-ODN was specific in that it reduced CpG DNA mediated activation of the HIV-1-LTR, but not activation mediated by LPS (FIG. 16A). Since even the CpG-containing PS-ODN were inactive in the RAW264 assay, the present inventors examined whether these might also be inhibitory. Indeed, SAAC-22 was also able to prevent activation by AO-1 and AAC-22 (not shown). These findings indicate that the phosphorothioate backbone has a dominant repressive effect on the ability of CpG DNA to activate in the short term assays.

The time course of action of PS-ODN specific blockade is investigated in FIG. 17. There appeared to be no requirement for pretreatment; in fact the extent of inhibition of NF-κB-dependent transcription was greatest when PS-ODN were co-administered, whereas there was little effect where it was added only 15 minutes after treatment with CpG PO-ODN (FIG. 17). The lack of inhibition following delayed addition is consistent with the pattern of activation of ERK-1/2 (see FIG. 15) which is already maximal after 20 minutes. High dose inhibition observed in the dose response curve for PS-ODN activation of NO production could also reflect a compromise between stimulation by the CpG motif, and inhibition by the backbone. This possibility was investigated by preincubating cells with non-CpG ODN. As observed in the RAW264-HIV-1-LTR assay, non-stimulatory PS-ODN specifically blocked the response to AO-1 or AOS-1, whereas no inhibition of the LPS response was observed (FIG. 18). By

contrast, there was no effect of the corresponding non-CpG PO-ODN (NAO-1) even at 10-fold excess. Note that the concentration of NAOS-1 that prevents responses to stimulatory DNA is in the same range that caused high dose inhibition, indicating that the two phenomena are probably related.

5.4 *CCGG motifs can stimulate macrophages and lack inhibitory activity*

The previous sections demonstrated that PS-ODN specifically prevent responses to CpG DNA, whereas non-stimulatory PO-ODN lack this activity. However, there is some evidence that specific PO-ODNs can inhibit responses to CpG DNA. Specifically, CCGG motifs have been reported to be inhibitory both *in cis* (when such a CCGG motif is included within the active ODN) and *in trans* (when the CCGG motif is present on another ODN co-adminstered with an active ODN) (Krieg *et al.*, 1998, Proc. Natl. Acad. Sci. USA **95** 12631). The present inventors considered the possibility that inhibition by PS-ODN and CCGG PO-ODN might be related. To address the actions of CCGG motifs we co-incubated cells with AO-1 and varying amounts of IO-2 (a PO-ODN based on AO-1 with the single GACGTT motif of AO-1 replaced with ACCGGT). FIG. 19A shows that no inhibition was observed with IO-2 even at a 30-fold molar excess over that of AO-1. FIG. 19A suggested that co-incubation of IO-2 with AO-1 was actually increasing the activity, therefore we investigated whether IO-2 was active in its own right. Indeed, although much less potent than AO-1, IO-2 did activate at high concentrations (FIG. 19B). Activation was not due to contaminating LPS as DNase I digestion abolished activity (FIG. 19C). As IO-2 did have activity, albeit low, it is speculated that such low level activation at moderate concentrations may induce a form of tolerance, possibly accounting for the inhibition reported by others. To address this, preincubation was performed with IO-2 at varying concentrations with subsequent AO-1 treatment. Even pretreatment with IO-2 at activating concentrations for 2 or 6 hrs did not reduce the subsequent CpG specific activation by AO-1 (FIG. 19D).

EXAMPLE 6

General Discussion

Based upon the results presented herein, the present inventors have shown that RPA is the receptor for immunostimulatory DNA. The first evidence that a relatively non-specific ssDNA binding protein such as RPA is the receptor is based upon reassessment of the sequence specificity of the response. In essence, the response of macrophages to immunostimulatory ODNs is not absolutely determined by CpG core motifs, and actually appears to involve a relaxed activation motif of up to 16 bases wherein the primary effect of substitutions is to shift the dose-response curve. Because of the logistics of carrying out substitutions of all possible bases even within a short 6 base core motif, there have been limited studies of sequence specificity of ODN responses, confused further by the effect of phosphorothioate backbone. The original report that a palindromic core was required for NK cell activation was not confirmed in studies on B cell mitogenesis (Krieg *et al.*, 1995, *supra*) and is not evident in any macrophage assays such as described herein.

The second line of evidence favouring RPA as receptor is that immunostimulatory DNAs induce in primary macrophages a range of changes that are characteristic of DNA damage in other cell types, including induction of NF-κB-dependent transcription and arrest of the cell cycle in G1. There is overwhelming evidence that RPA is a recognition molecule in several forms of DNA damage (Wold, 1997, *supra*). The present inventors have introduced novel cell-based assays for identifying immunostimulatory DNA: the rapid down-regulation of the CSF-1 receptor (*c-fms*); and prevention of cell cycle progression from G_1 to S phase.

The former reflects a massive increase in vesicle trafficking that occurs in stimulated macrophages and can be seen in rapid polar spreading of the stimulated cells on the substratum (not shown). The down-modulation is specific to the CSF-1 receptor in that the complement receptor (CD11b) and the macrophage-specific F4/80 antigen were not down-

modulated in the same time frame (data not shown). It may also be that down modulation generates some form of stimulation via the CSF-1 receptor that is, in turn, connected to the anti-apoptotic response to both DNA and LPS. Low amounts of the receptor ligand, CSF-1 itself, can maintain viability of macrophages without triggering cell cycle progression (Roth & Stanley, 1992, Curr. Top. Microbiol. Immunol. **181** 141). Regardless of the causal relationship, and the mechanism of selective down-modulation, disappearance of CSF-1R provides a rapid and sensitive assay for immunostimulatory ODNs (and LPS).

Thirdly, complementing the evidence that the sequence specificity of the biological response to ODNs is relatively relaxed, and even that the core CpG is not absolutely required, we show that RPA has sequence specificity for binding to DNA which correlates with relative biological efficacy in macrophages. RPA can distinguish activating from non-activating or methylated ODN, and ODN with subtly differing activity in the RAW264a4 assay. RPA has been reported previously to have a 50-fold selective affinity for poly-pyrimidines as opposed to poly-purines (Wold, 1997, *supra*). Solution of the crystal structure of RPA70 complexed with an oligo-dC 8mer (Bochkarev *et al.*, 1997, Nature **385** 176) revealed a core binding site comprising the two ssDNA-binding domains in asymmetric conformation. Each ssDNA binding domain contacts 3 nucleotides, and the space between the two domains is bridged by two nucleotides. Both domains form multiple hydrogen bonds with all the DNA bases, implying potential for DNA-sequence-specific binding. The two nucleotides that span the gap between the two ssDNA-binding domains make 6 independent hydrogen bonds with the protein, including 4 with the bases. The hydrogen bonds with these nucleotides are the same as would be formed in a base pair, involving O2 and N4 in each case. Given that the critical CpG is centred in the optimal sequence for immunostimulatory oligonucleotides, the present inventors hypothesise that the active CpG would form the two spanning nucleotides in the active complex with RPA and that the

asymmetry of the two ssDNA binding domains in the complex could explain the asymmetry of the optimal immunostimulatory sequence RRCGYY. Further molecular modelling studies may provide insight into the possible effect of methylation of a bound cytosine in this proposed structure and the possible effect of contacts with the two bridging bases on the conformation of RPA70. Of course, as discussed below, the full complex with RPA also involves contact with RPA34, which could explain the more extended 16bp optimal motif indicated by the biological and DNA binding data. Thus, CpG sequence specificity and the reduced affinity for ODNs containing methylated C is entirely conceivable based upon the structure of RPA70.

The final evidence supporting the identity of RPA as the receptor is that it is abundant in the cytoplasm. Furthermore, there is no other detectable candidate receptor for DNA under our conditions of assay. In the cell fractionations shown in FIG. 1, a nuclear protein of comparable size to RPA, Oct1, was completely restricted to the nuclear fraction under similar conditions. A complex as large as RPA is probably not expelled from the nucleus under the conditions of mild cell lysis with NP-40 when other nuclear proteins are retained. The location of RPA in the nucleus has been inferred based upon immunohistochemical localisation. However, there are contrary published data obtained from experiments using antibodies against different subunits; RPA14 was clearly detected in the cytoplasm (Wold, 1997, *supra*). Immunocytochemistry may give a false impression based upon the particular concentration of protein in the nucleus, leakage or selective destruction of antigen under the conditions of immunohistochemical localisation, or obscuring of antigenic epitopes in specific complexes of cytoplasmic RPA with other proteins (see below).

An important aspect of the work presented herein is the identification of at least three forms of RPA in macrophages and B cells. The UV cross-linking indicates that band A (in the mouse) contains both RPA 70 and RPA34, and that both subunits contact DNA. As noted in the introduction, RPA is known to undergo a conformational change upon

binding of ssDNA. Initial binding of RPA is proposed to involve the approximate 8 base "footprint" seen in the 3D structure (Wold, 1997, *supra*) and to be relatively low affinity. Interactions between the RPA 34 and RPA14 subunits (also known as RPA2 and RPA3 respectively), which have independent ssDNA binding activity and the C-terminus of RPA70 (RPA1; Wold, 1997, *supra*) are required for transition to a high affinity irreversible binding state. Based upon the irreversibility of binding, the UV cross-linking and the inhibitory effect of Mg^{2+} (which is known to affect the conformational change in RPA) the present inventors propose that band A is the end-stage high affinity conformation of RPA. The conformation difference may explain why Band A did not react with all of the particular anti-RPA antibodies available to the present inventors. Others (Seroussi & Lavi, 1993, *supra*) have found that the slowest migrating RPA band was bound by a different anti-RPA70 antibody which failed to recognize the faster migrating band(s) containing proteolytically cleaved RPA.

In the band B complex in FIG. 9, which can be cold competed by oligonucleotide added after binding of label only the 70kD RPA subunit appears to be in sufficiently close proximity to the ODN to permit UV cross-linking, but the complex is supershifted by anti-RPA34 antibody. One basis for the difference between RPA in band A and band B would be the phosphorylation of RPA 34 which is known to be occur upon DNA binding (Wold, 1997, *supra*) and might conceivably alter subsequent binding activity so that the high affinity complex involving contact with RPA34 cannot be formed. Band C, the complex containing the 55kD putative proteolytic cleavage product of RPA70 also showed no evidence of DNA contact with RPA 34. Initially, band C was observed inconsistently in macrophage extracts. Others have suggested that RPA70 cleavage may occur following cell extraction as an explanation for inconsistent identification of a presumed equivalent of our band C (Seroussi & Lavi, 1993, *supra*). However, the present inventors found that when extracts are prepared fresh and the incubation is carried out with added calcium, the level of band C in

cytoplasmic extracts is consistent and reproducible (not shown). Accordingly, it has been concluded that band C is normally present in macrophages and represents a novel functional form of RPA.

Interest in studying the responses to CpG-containing DNA arises from several distinct biological applications. In the context of gene therapy, DNA vaccines and the possible roles of CpG DNA sequences in host-pathogen interactions, the active molecules of interest are native phosphodiester DNAs. Conversely, applications of CpG DNA in immunotherapy are based upon phosphorothioate-modified backbones, and PS-ODNs are also commonly applied in anti-sense therapies. In this study we have shown that PS-ODNs have quite distinct biological activities from PO-ODNs in modulating CpG driven responses.

The induction of NO production in interferon-γ-primed macrophages has proven to be a robust and convenient assay of the actions of CpG DNA. In this assay, PS-ODNs containing a single CpG motif were much more active than their PO-ODN counterpart, but the response was still abolished by a GpC transversion in the core motif. The dose-response for CpG PS-ODNs commonly exhibited an optimal concentration for maximal activity, and the response declined at higher concentrations (FIGS. 11a and 11b). High dose inhibition of NO induction is probably not due to intrinsic toxicity but reflects specific inhibition of the response to stimulatory DNA. Pretreatment of RAW264 cells with non-stimulatory PS-ODN prevented subsequent activation by stimulatory PO-ODN, CpG containing PS-ODN and bacterial DNA (data not shown), but did not block activation by LPS (FIG. 17).

By contrast to the higher activity in the iNOS and cell viability assays, in the HIV-LTR and CSF-1R down-modulation assays, PS-ODN were much less active than PO-ODN. As in the longer assays, PS-ODN specifically blocked responses to PO-ODN or bacterial DNA. In effect, the phosphorothioate backbone auto-inhibits response to CpG motifs. The lack of activity in the shorter assays is probably a reflection of slower time

courses. Activation of NF-κB in macrophages is transient and luciferase is unstable (Sweet *et al.*, 1997, *supra*). With a fast-acting agonist such as PO-ODN or LPS, there is a relatively synchronous activation of cells in the population so that there is a large peak of luciferase activity after 2 hrs. The low level activation seen in response to PS-ODN probably reflects asynchronous activation of smaller subpopulations of cells at any particular time. This hypothesis is supported by the pattern of down-modulation of CSF-1R (FIG. 14) which occurred in only 25% of cells after 2 hrs. Stimulation of the MAPKinases ERK-1, and ERK-2, a rapid response to both LPS and CpG DNA in CSF-1-starved BMM, also occurred much more slowly in response to PS-ODN (FIG. 14D). The fact that it did occur eventually is consistent with the putative role of ERK-1/2 in CSF-1 signal transduction leading to the maintenance of cell viability. ERK-1/2 are the major kinases responsible for phosphorylation of the transcription factor Ets-2 in macrophages (Fowles *et al.*, 1998, Mol. Cell. Biol. **18** 5148; Stacey *et al.*, 1995, Mol. Cell. Biol. **15** 3430)), which in turn activates transcription of several target genes associated with cell survival including Bcl-x_L (Sevilla *et al.*, 1999, Mol. Cell. Biol. **19** 2624).

By contrast to PS-ODN, non-stimulatory PO-ODN (GpC transversion) did not prevent activation by CpG DNA (FIG. 15B). Others have reported on the existence of inhibitory CpG DNA sequences containing a core CCGG motif (Krieg *et al.*, 1998, *supra*) but even preincubation with a large excess of CCGG-containing ODN failed to reduce the response to subsequent stimulation by optimal activating sequences (FIG. 18). In fact, the CCGG sequence was biologically active, simply requiring higher concentrations than an optimal motif to activate the HIV-1-LTR in RAW264 cells and in the iNOS assay (not shown). This confirms that the major effect of altered sequences flanking the core CpG motif is to change the dose response curve.

Another group has reported that non-stimulatory PS-ODN can prevent the activation of murine dendritic cells by an active ODN, and has

inferred that the mechanism involves saturation of a non-selective cellular uptake pathway for DNA (Hacker *et al.*, 1998, *supra*). The uptake mechanism is likely to differ between cell types. T cells internalise ODN poorly. In murine bone marrow B cells, the uptake of oligonucleotides involved saturable binding to a cell surface receptor that also bound polyanions such as dextran sulphate (Zhao *et al.*, 1994, Blood **84** 3660). The process was regulated by stimuli such as LPS. The integrin, Mac-1, has been implicated as a specific receptor for ODN in neutrophils (Benimetskaya *et al.*, 1997, Nat. Med. **3** 414) but the present inventors found no evidence to support this function in murine macrophages, in which ODN did not alter the level of Mac-1 antigen detectable on the cell surface. In a number of other cell systems ODN at concentrations above 1 μM was internalised primarily by fluid-phase endocytosis (Yakubov *et al.*, 1989, Biochem. **86** 6454; Beltinger *et al.*, 1995, J. Clin. Invest. **95** 1814), which is inherently unlikely to become saturated by ligand. Given the intrinsically high endocytic rate of macrophages, the fluid phase pathway probably predominates in this cell type. In fact, one of the earliest effects of LPS and CpG DNA on macrophages is a profound spreading response and, in the case of LPS at least, increased vesicle trafficking and macropinocytosis (Poussin *et al.*, 1998, J. Biol. Chem. **273** 20285; Peppelenbosch *et al.*, 1999, Blood **93** 4011) which is probably associated in part with the rapid internalisation of the CSF-1R studied herein. Hence, CpG DNA is likely to increase its own uptake into macrophages leading to a self-amplifying cascade in response to stimulatory sequences. The lack of any inhibitory effect of a large excess of PO-ODN in this system indicates that the cellular uptake process is not saturated at concentrations of DNA that generate a maximal biological response and strongly supports the view that the determinant of CpG specificity lies within the cell.

What is the basis for the different behaviour of PS-ODN and PO-ODN? Studies on trafficking of ODNs in a number of cell systems have highlighted differences. In HL-60 human myeloid cells PS-ODNs appeared

to be mobilised to an acidic compartment, whereas PO-ODN were not, but this differential trafficking was also affected by sequence variations (Tonkinson & Stein, 1994, Nucl. Acid. Res. **22** 4268). In the same cells, PS-ODN, and to a lesser extent PO-ODN, blocked uptake of a pinocytotic marker, probably by stimulating exocytosis via inhibition of protein kinase C (Stein *et al.*, 1993, Biochemistry **32** 4855). Such a mechanism could permit PS-ODN to block the uptake of PO-ODN or bacterial DNA without competing directly for binding to a receptor. However, Chu *et al.*, 1999, J. Immunol. 163 1188) observed only a small reduction of fluid-phase uptake of FITC-dextran in activated peritoneal macrophages treated overnight with PS-ODN, or LPS. The mechanisms by which ODNs are released from the endocytic compartment to the cytosol are not well understood. Endosomal release could be a rate limiting step for responses to CpG DNA irrespective of the cellular accumulation of ODN *per se*. It may be this step that occurs more slowly with PS-ODN. If the release of ODN into the cytoplasm is limiting and saturable, non-stimulatory PS-ODN are likely to accumulate to greater levels in the endosomal compartment because of their greater stability and hence will be more effective competitive inhibitors of release than PO-ODN. In keeping with a limiting role at this stage, transfection reagents have been found to increase the efficacy of ODN-mediated cellular responses in a number of systems (Thierry & Dritschilo, 1992, Nucl. Acid. Res. **20** 5691; Sonehara *et al.*, 1996, J. Interferon Cytokine Res. **16** 799).

The hypothesis that PS-ODN act in part at the level of CpG recognition was supported by studies of the putative receptor for CpG DNA, RPA. PS-ODN at low concentration were able to compete for binding of PO-ODN to RPA under the conditions of EMSA. The affinity of competition was so high as to preclude realistic assessment of CpG DNA specificity; even non-CpG PS-ODN were at least as effective as self CpG PO-ODN in cold competition. The ability of RPA to distinguish CpG motifs must require alignment of oligonucleotide in such a way that the CpG dinucleotide can be recognised by a specific secondary recognition moiety within RPA or

another protein. It may be this secondary recognition that is critical for initiation of a biological response. Structural studies of RPA indicate the presence of at least two DNA-binding sites, one within the 70 kD subunit and one formed by the 32 kD and 14 kD subunits. Binding probably involves a two-stage process where the 70 kD subunit, which possesses much higher affinity binding activity in isolation, makes the first contact and secondary contact is made subsequently with the 32 kD/14 kD subunits or with another component, such as the zinc fingers. A very high affinity initial interaction of PS-ODN with RPA70 is likely to inhibit dissociation and "scanning" to permit secondary recognition of the CpG dinucleotide. Hence, correct binding of CpG leading to activation would occur solely as predicted by chance (i.e. in 1 complex in 20 with a 20base ODN). It should be noted that such scanning also must occur in the binding of a PO-ODN. The complexes seen in EMSA are likely to be a mixture of random lower affinity complexes, where the CpG is not aligned, and higher affinity correctly aligned complexes. This pattern obscures the full extent of differential binding affinity of RPA for CpG.

The response to PS-ODN is complex because their intrinsic lack of effectiveness is compensated in part by their greater stability. The prediction of the above model is that in PS-ODNs, inclusion of an additional CpG motif, perhaps with appropriate spacing to another motif allowing simultaneous binding by more than one RPA molecule, could amplify the probability of response exponentially. Aside from sequence changes, optimisation of stimulatory PS-ODNs requires a compromise between optimising uptake and stability, which may be favoured by longer ODNs, and reducing the number of inhibitory phosphorothioate linkages in the backbone. Assuming uptake can be optimised, it should be possible to modulate that balance by reducing the length of the PS component of the ODN to the known "footprint" of RPA (8 bases), increasing further the number of CpG motifs per unit length, producing ODNs with mixed backbones, or identifying backbone modifications that do not generate inhibitory activity (which could be tested using the assays disclosed herein).

Chimaeric immunostimulators can also be envisaged, in which the stable PS-ODN core is flanked by signals, such as high mannose sugars, that can fulfil the function of expediting uptake and access to RPA.

In summary, the present inventors have provided evidence that there are several forms of RPA in macrophages, one or all of which have characteristics consistent with identity as the cytoplasmic receptor for immunostimulatory DNA. This conclusion is based upon the sub-cellular location of RPA, the binding specificity of RPA and the nature of the biological response to immunostimulatory DNA. A fundamental understanding of the recognition of immunostimulatory DNA by different forms of RPA should permit a more rational structure-function approach to optimising immunostimulatory DNA as a therapeutic agent.

The present inventors have also demonstrated that the relative efficacy of immunostimulatory DNA in macrophage activation varies depending upon the assay system. It has also been shown that the phosphorothioate modification specifically interferes with some actions of CpG DNA, so that PS-ODNs are intrinsically self-inhibitory. This observation could lead to development of improved reagents to exploit the therapeutic potential of immunostimulatory DNA in a wide range of human disease states.

It will be understood by the skilled person that the invention is not limited to the particular embodiments described in detail herein, but also includes other embodiments consistent with the broad spririt and scope of the invention.

CLAIMS

1. A method of detecting immunostimulatory DNA, including the steps of:-

(i) combining isolated RPA with a sample containing DNA;

(ii) forming a complex between said isolated RPA and immunostimulatory DNA if present in said sample; and

(iii) detecting said complex formed at step (ii).

2. A method of identifying an immunostimulatory DNA agonist or antagonist, including the steps of:-

(i) combining isolated RPA with a sample suspected of containing an immunostimulatory DNA agonist or antagonist; and

(ii) determining whether or not a complex forms between said isolated RPA and said agonist or antagonist, formation of a complex indicating that said agonist or antagonist is present in said sample.

3. The method of Claim 2, wherein an immunostimulatory DNA is added at step (i) so that at step (ii) said agonist or antagonist prevents formation of a complex between said immunostimulatory DNA and said isolated RPA.

4. The method of any one of Claims 1-3, wherein the RPA complex, if formed at step (ii), is detected by EMSA.

5. The method of Claim 4, wherein an anti-RPA antibody is included at step (ii).

6. A kit for detecting immunostimulatory DNA, said kit comprising:-

(i) one or more isolated RPA subunits; and

(ii) an RPA-specific antibody.

7. The kit of Claim 6, wherein the isolated RPA subunits include a 70kD subunit, a 34 kD subunit and a 14 kD subunit and, optionally, a 55 kD proteolytic fragment of the 70 kD subunit.

8. A method of detecting an immunostimulatory DNA including the steps of:-

(i) combining macrophage cells with a sample suspected of containing immunostimulatory DNA;

(ii) measuring a response by said macrophage cells to said immunostimulatory DNA if present in said sample, said response selected from the group consisting of:-

(a) a cell-cycle arrest; and

(b) a reduction in CSF-1 receptor (CSF1-R) expression.

9. A method of identifying an immunostimulatory DNA antagonist, including the steps of:-

(i) combining macrophage cells with immunostimulatory DNA and a sample suspected of containing an immunostimulatory DNA antagonist; and

(ii) measuring a response, or an absence of said response, by said macrophage cells to said immunostimulatory DNA, said response selected from the group consisting of:-

(a) a cell-cycle arrest;and

(b) a reduction in CSF-1 receptor (CSF1-R) expression;

wherein said absence of a response is indicative of said antagonist being present in said sample.

10. A method of identifying an immunostimulatory DNA agonist, including the steps of:-

(i) combining macrophage cells a sample suspected of containing an immunostimulatory DNA agonist;

(ii) measuring a response by said macrophage cells selected from the group consisting of:-

(a) a cell-cycle arrest;and

(b) a reduction in CSF-1 receptor (CSF1-R) expression.

wherein said response is indicative of said agonist being present in said sample.

11. The method of any one of Claims 8-10, wherein the cell cycle arrest is at G_1-S phase transition.

12. The method of any one of Claims 8-10, wherein the reduction in CSF-1R expression is detected by cell surface immunofluorescence.

13. An immunostimulatory DNA identified by the method of Claim 1 or Claim 8.

14. An immunostimulatory DNA agonist identified by the method of any one of Claims 2, 3 or 9.

15. An immunostimulatory DNA antagonist identified by the method of any one of Claims 2, 3 or Claim 10.

16. An immunostimulatory DNA antagonist which comprises a DNA having one or more phosphorothioate linkages.

17. The antagonist of Claim 16, wherein the immunostimulatory DNA is a CpG-containing DNA having a phosphodiester backbone.

18. A therapeutic or prophylactic composition comprising the immunostimulatory DNA of Claim 13, together with an acceptable carrier or delivery agent.

19. A therapeutic or prophylactic composition comprising the immunostimulatory DNA agonist of Claim 14, together with an acceptable carrier or delivery agent.

20. A therapeutic or prophylactic composition comprising the immunostimulatory DNA antagonist of any one of Claims 15-17, together with an acceptable carrier or delivery agent.

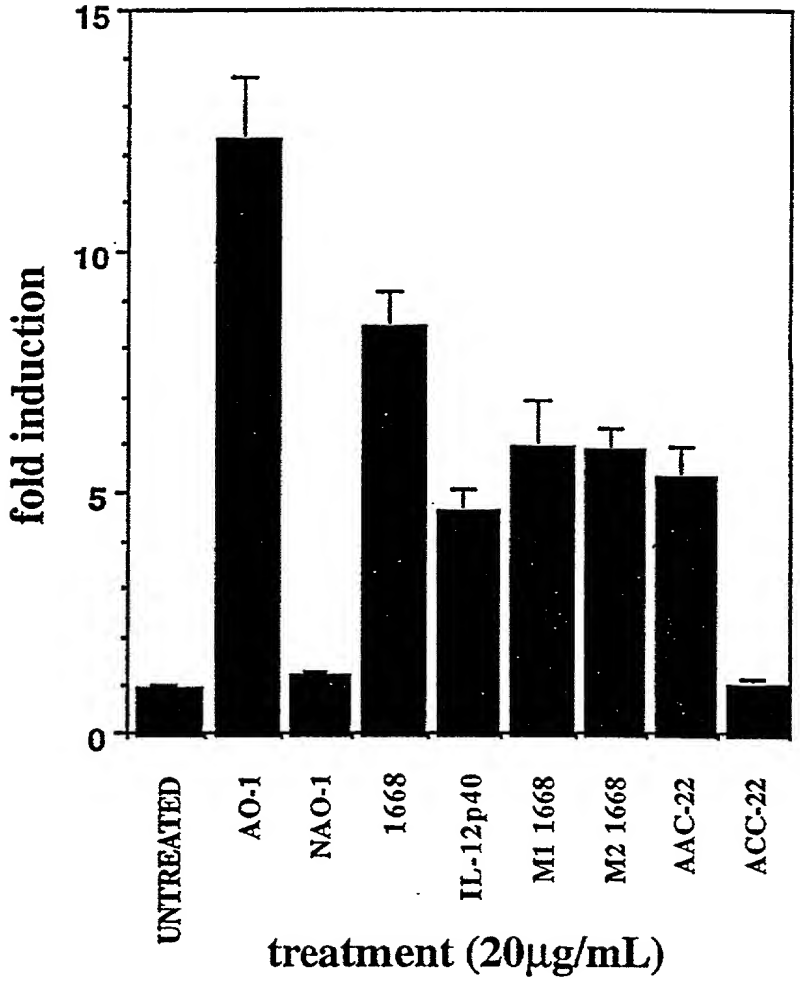

15
10
5
0
fold induction
UNTREATED
AO-1
NAO-1
1668
IL-12p40
M1 1668
M2 1668
AAC-22
ACC-22
treatment (20µg/mL)

FIG. 1A

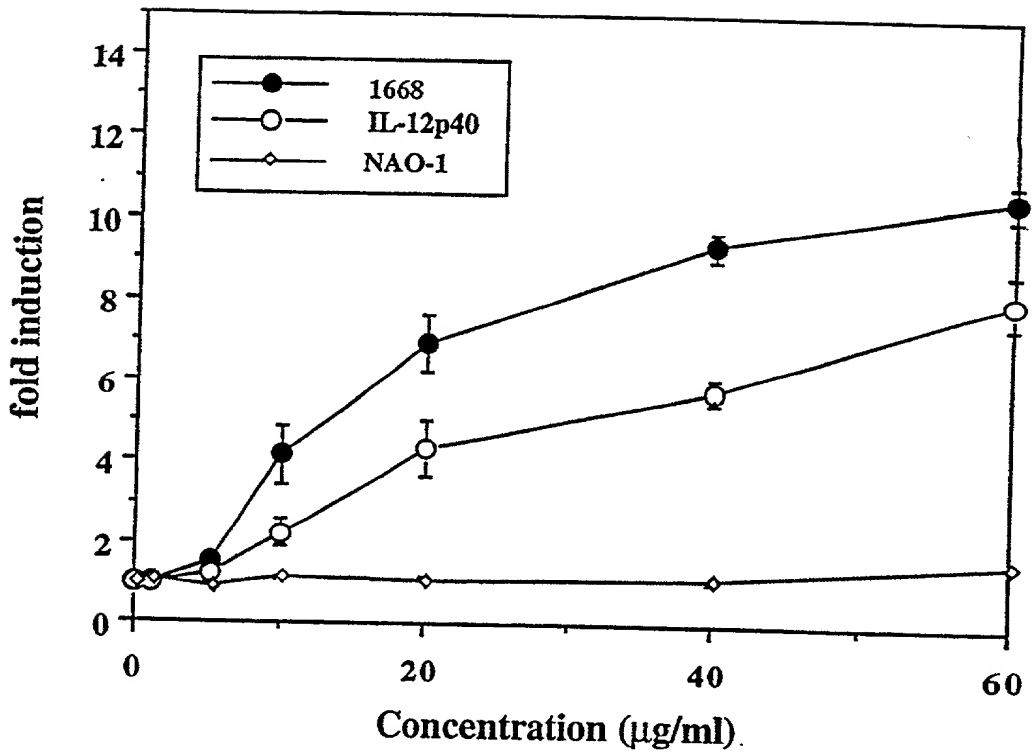

1668
IL-12p40
NAO-1
fold induction
Concentration (µg/ml)
0
2
4
6
8
10
12
14
0
20
40
60

FIG. 1B

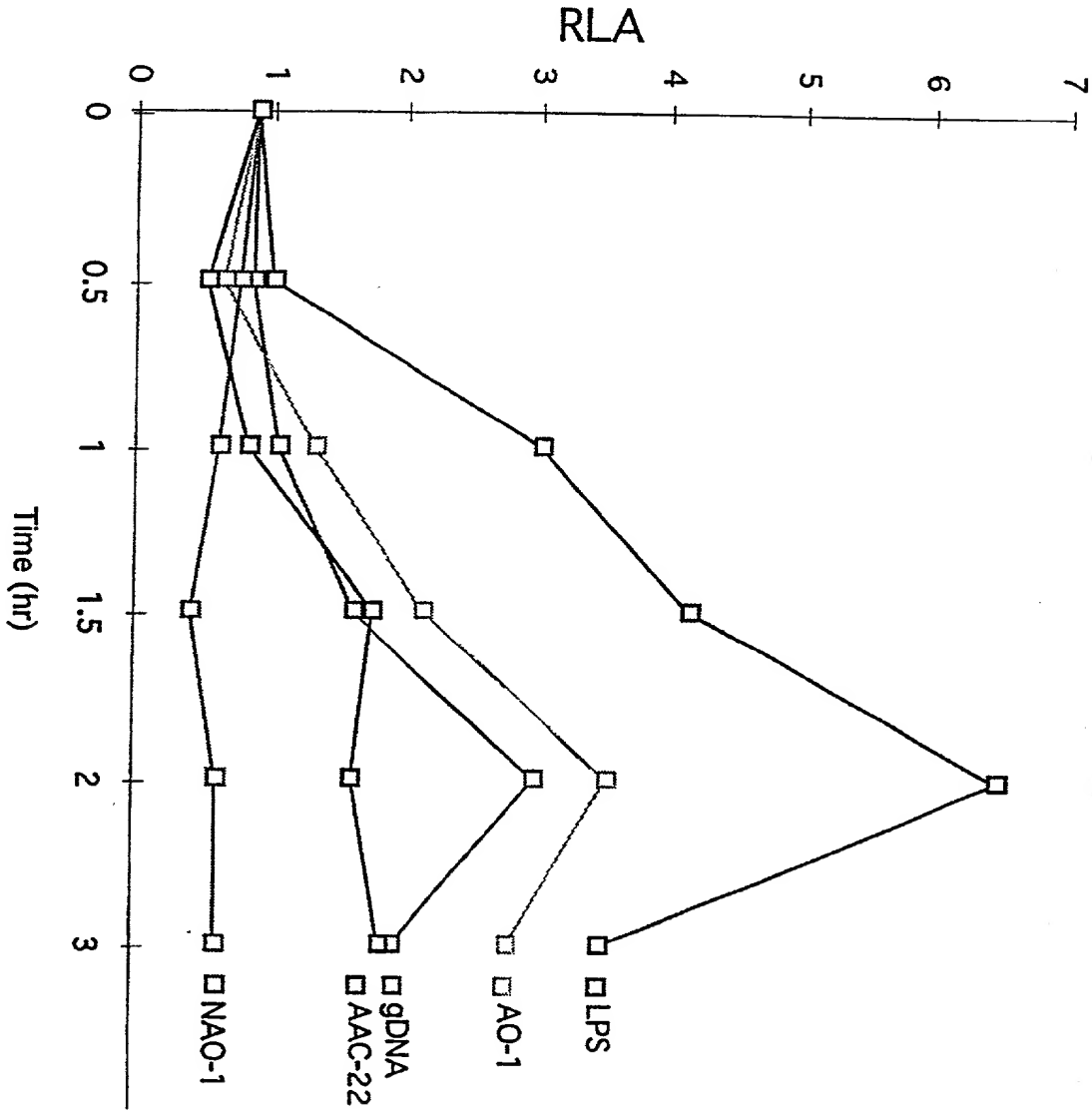


FIG. 1C

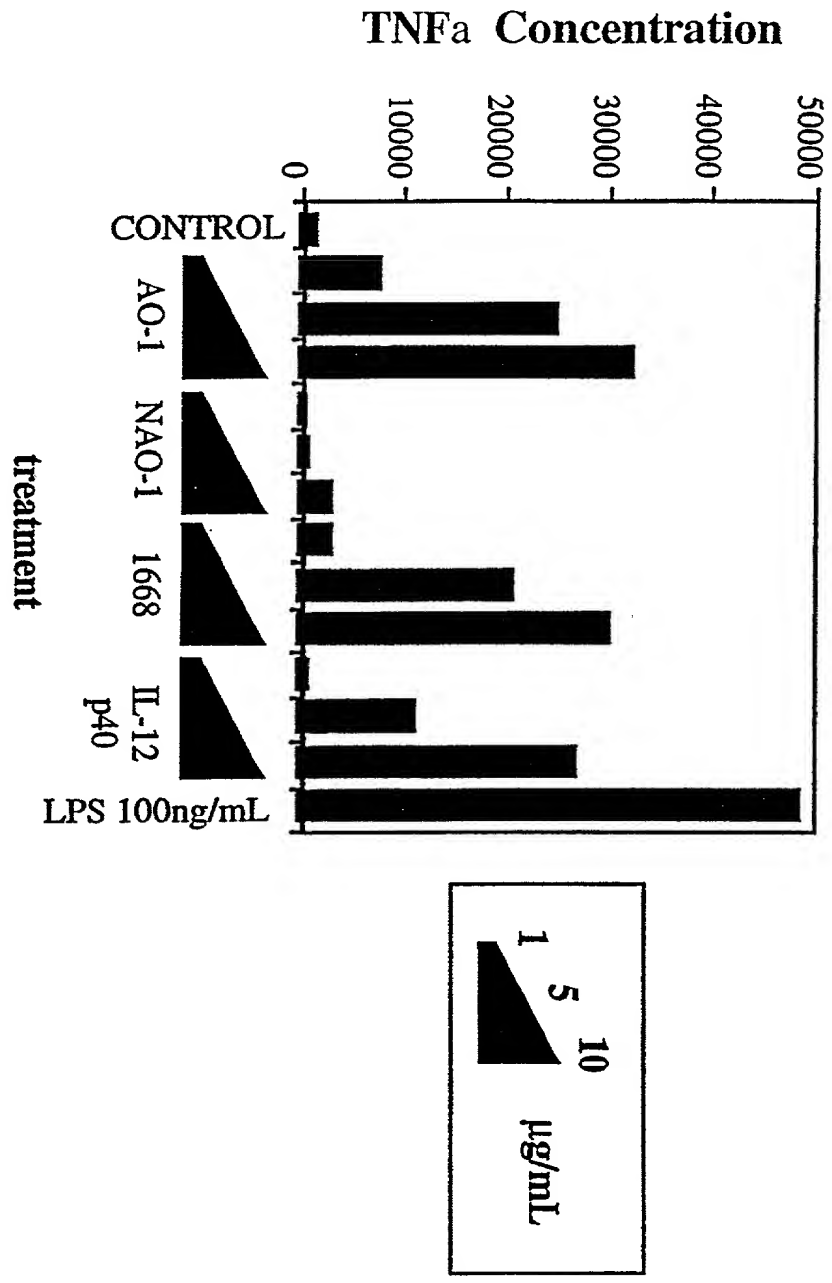
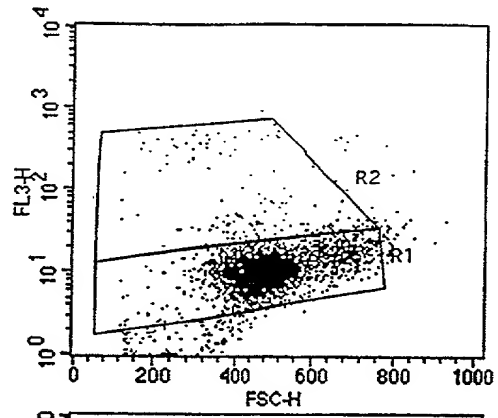

TNFa Concentration
0
10000
20000
30000
40000
50000
CONTROL
AO-1
NAO-1
1668
IL-12 p40
LPS 100ng/mL
treatment
1
5
10
µg/mL

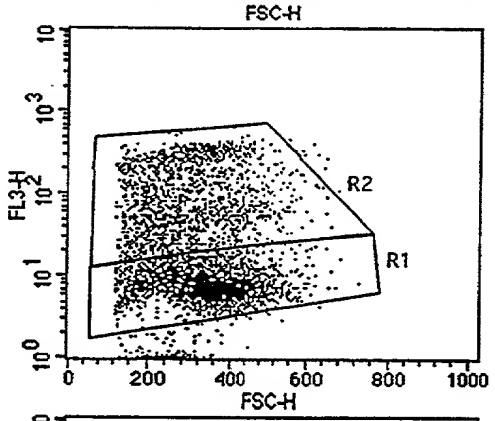
FIG. 2



+CSF-1

Region Statistics

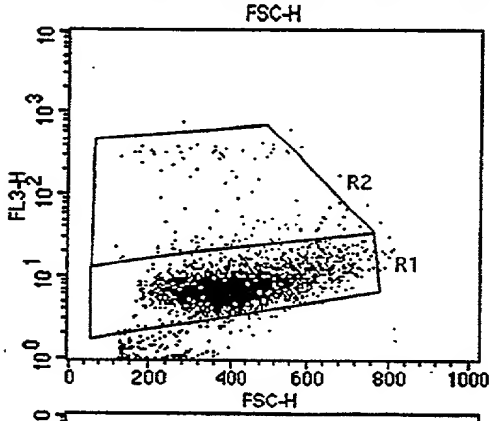
Region		% Gated
R1	Viable	95.6
R2	Non-Viable	4.4



- CSF-1

Region Statistics

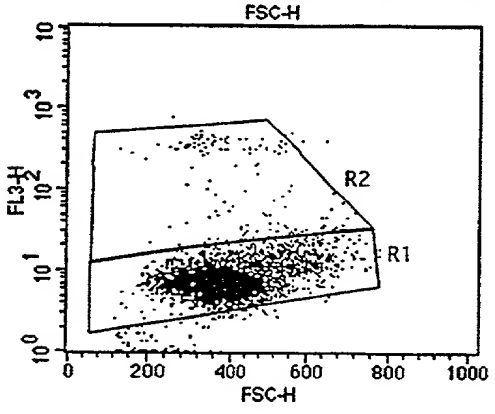
Region		% Gated
R1	Viable	59.3
R2	Non-Viable	60.7



- CSF-1 +DNA

Region Statistics

Region		% Gated
R1	Viable	96.9
R2	Non-Viable	3.1



- CSF-1 +LPS

Region Statistics

Region		% Gated
R1	Viable	96.5
R2	Non-Viable	3.5

FIG. 3

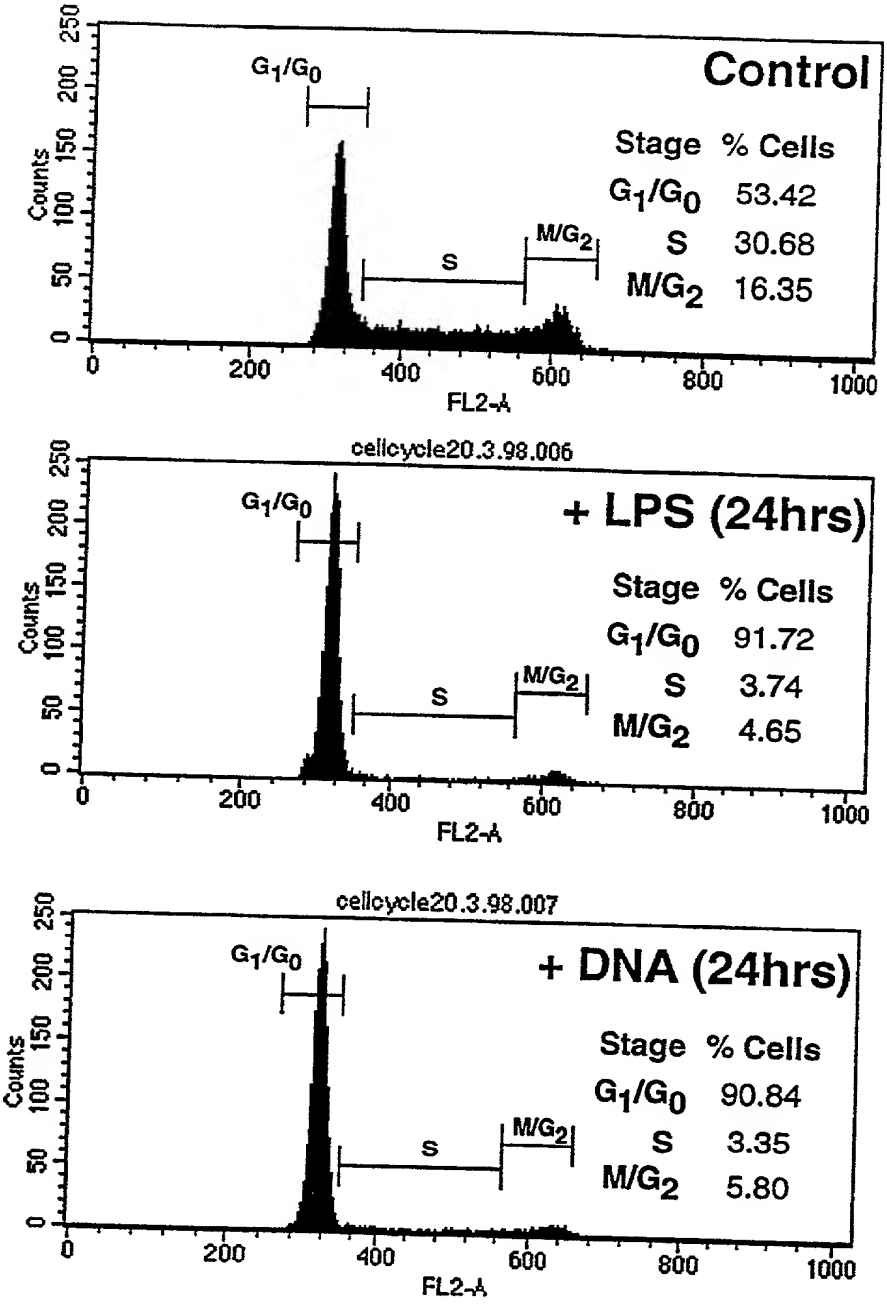

Control
Stage % Cells
G1/G0 53.42
S 30.68
M/G2 16.35
G1/G0
S
M/G2
Counts
FL2-A
cellcycle20.3.98.006
+ LPS (24hrs)
Stage % Cells
G1/G0 91.72
S 3.74
M/G2 4.65
cellcycle20.3.98.007
+ DNA (24hrs)
Stage % Cells
G1/G0 90.84
S 3.35
M/G2 5.80

FIG. 4

FIG. 5A

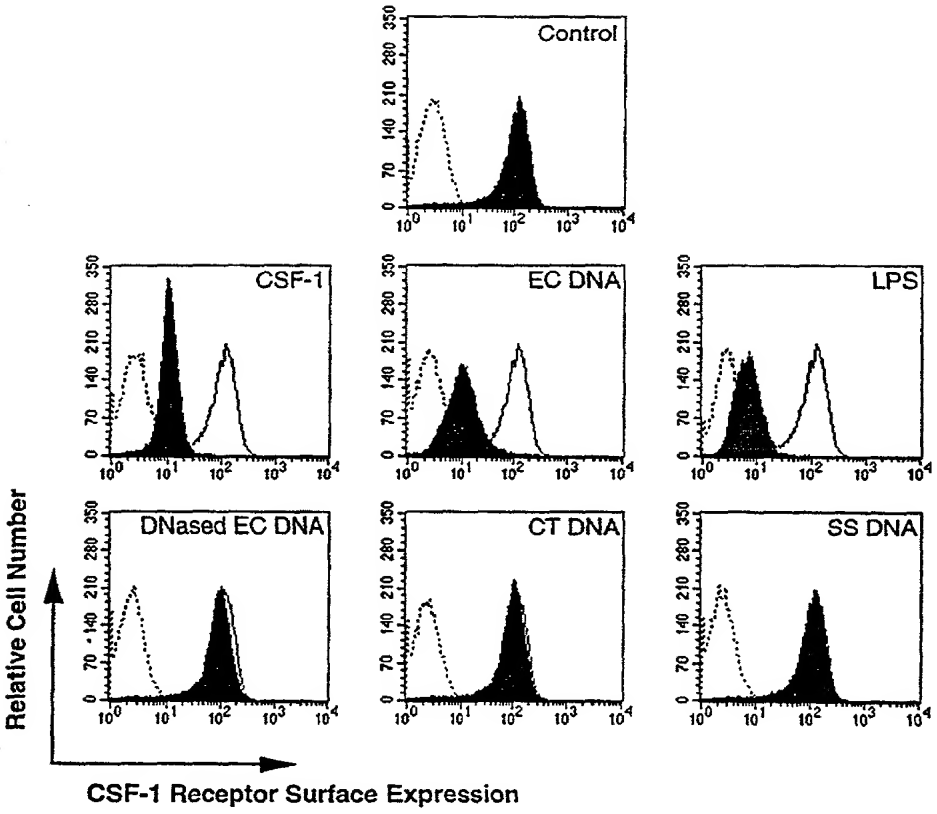

Control
CSF-1
EC DNA
LPS
DNased EC DNA
CT DNA
SS DNA
Relative Cell Number
CSF-1 Receptor Surface Expression

FIG. 5B

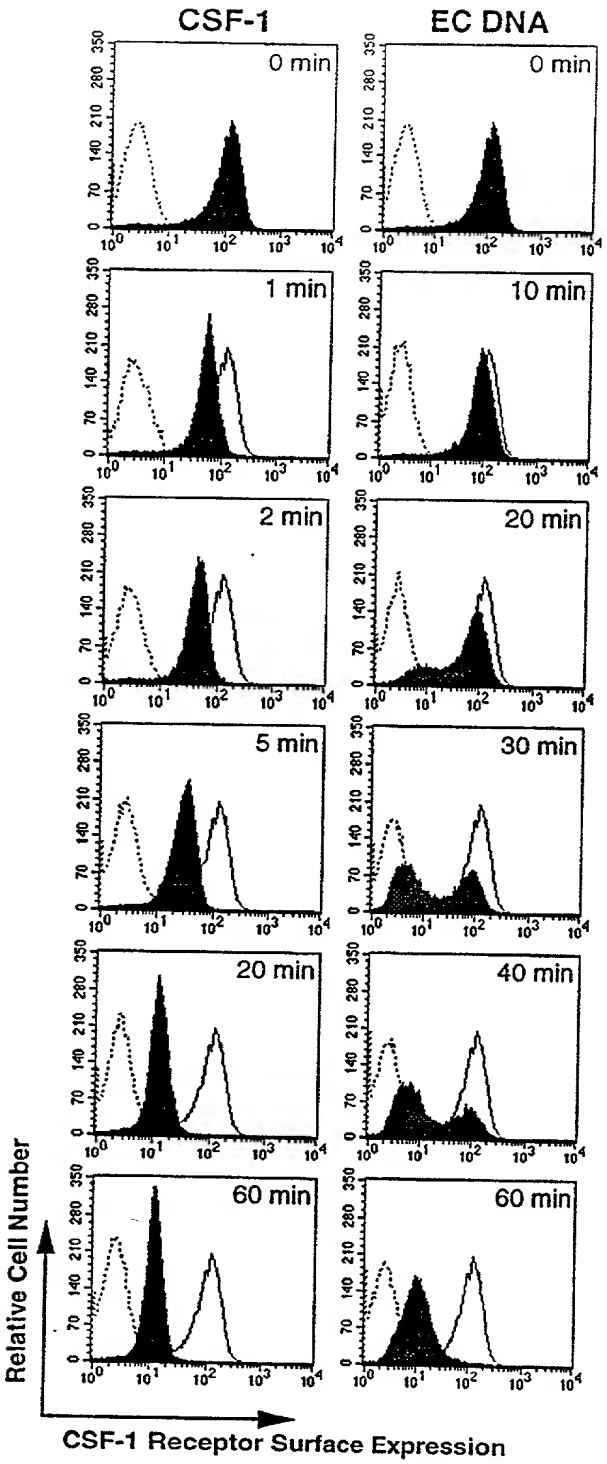

CSF-1
EC DNA
0 min
0 min
1 min
10 min
2 min
20 min
5 min
30 min
20 min
40 min
60 min
60 min
Relative Cell Number
CSF-1 Receptor Surface Expression

FIG. 5C

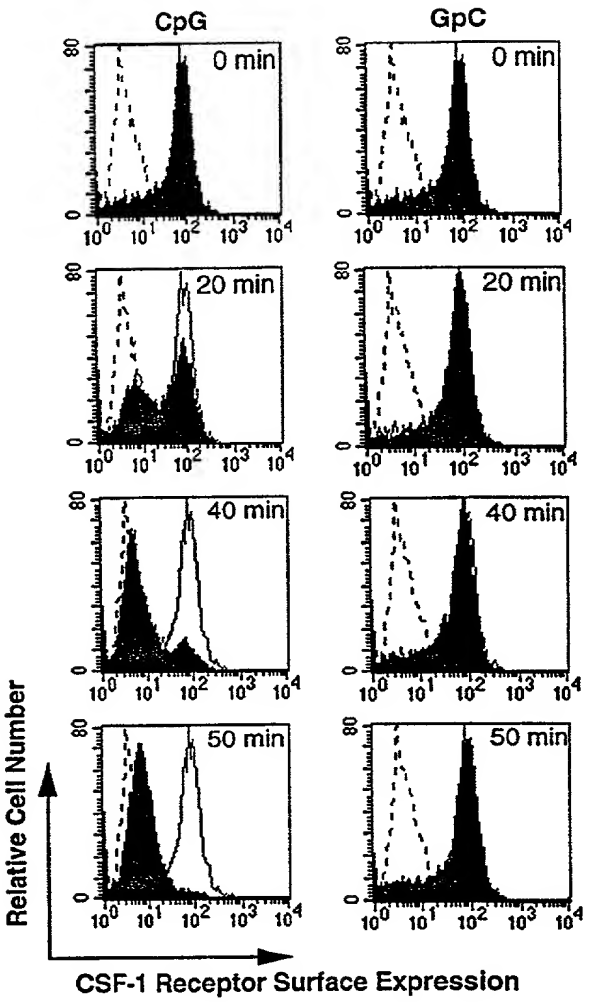

CpG
GpC
0 min
20 min
40 min
50 min
Relative Cell Number
CSF-1 Receptor Surface Expression

FIG. 5D

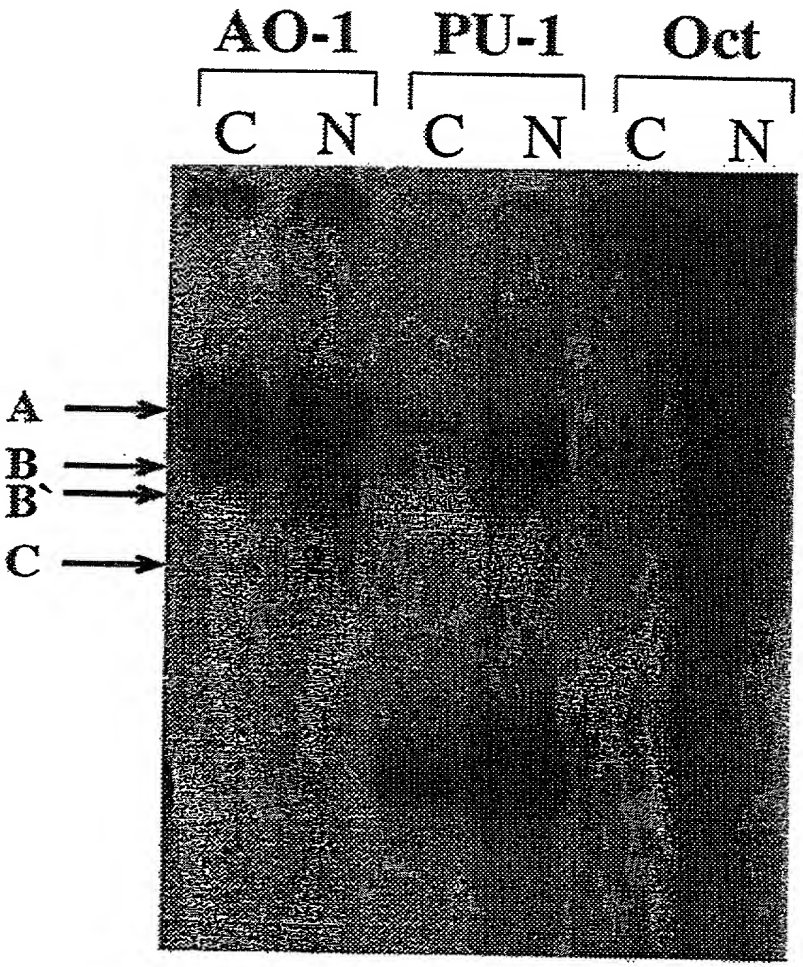


FIG. 6A

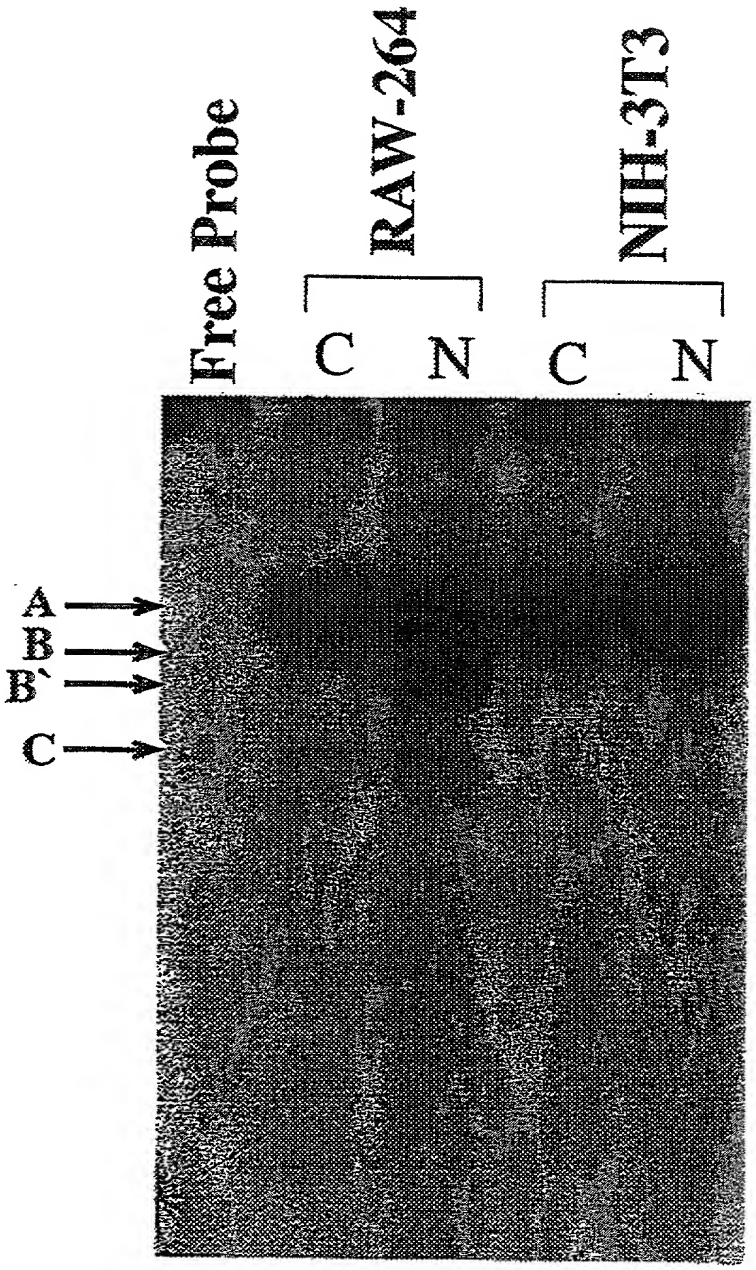


FIG. 6B

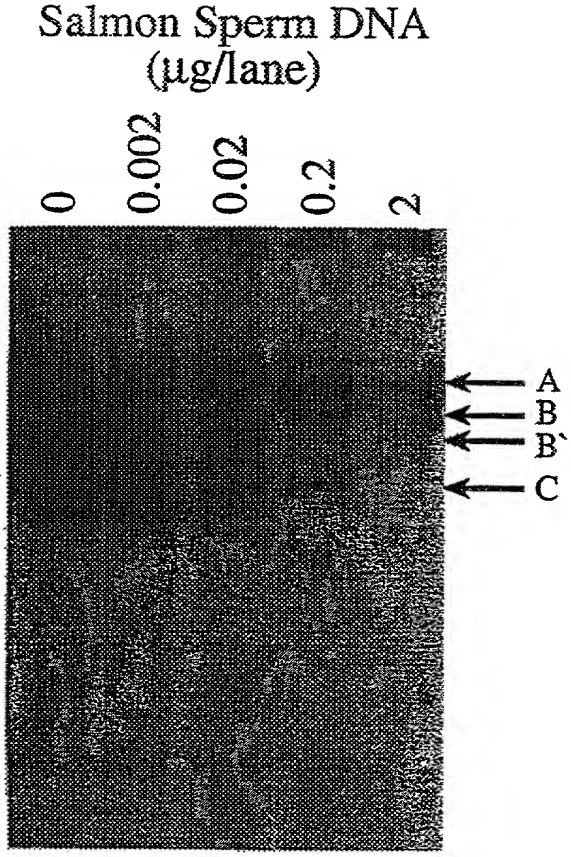


FIG. 6C

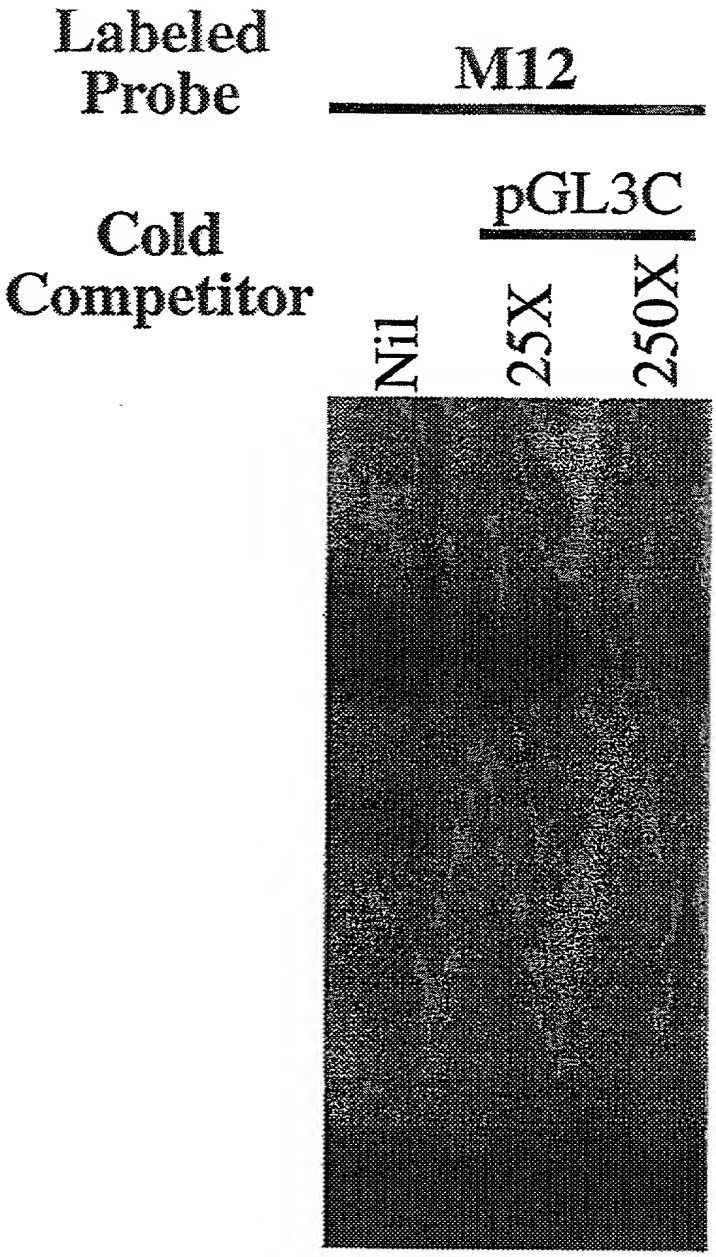


FIG. 6D

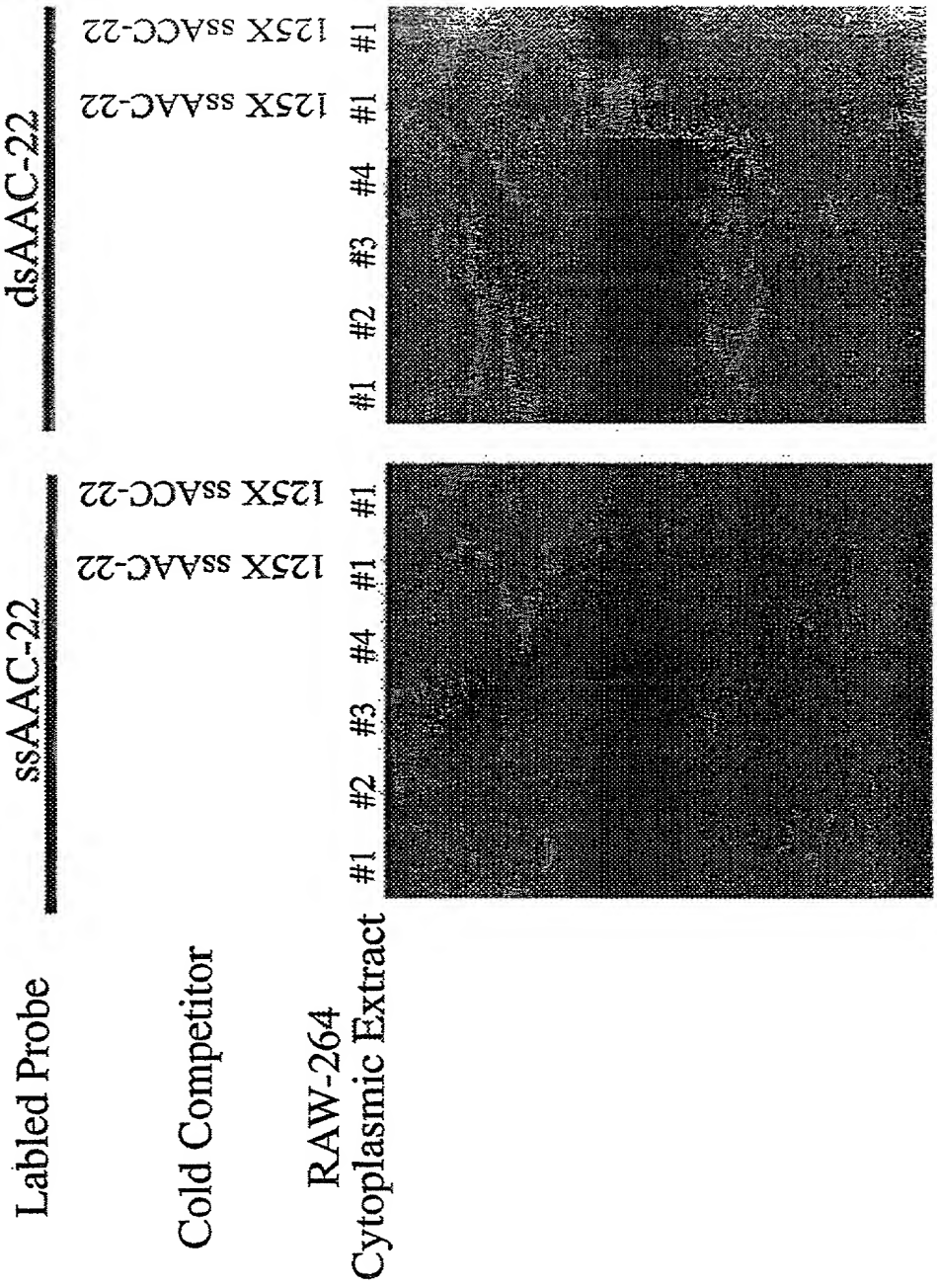

Labled Probe
ssAAC-22
dsAAC-22
Cold Competitor
125X ssAAC-22
125X ssACC-22
RAW-264
Cytoplasmic Extract
#1
#2
#3
#4
#1
#1

FIG. 7A

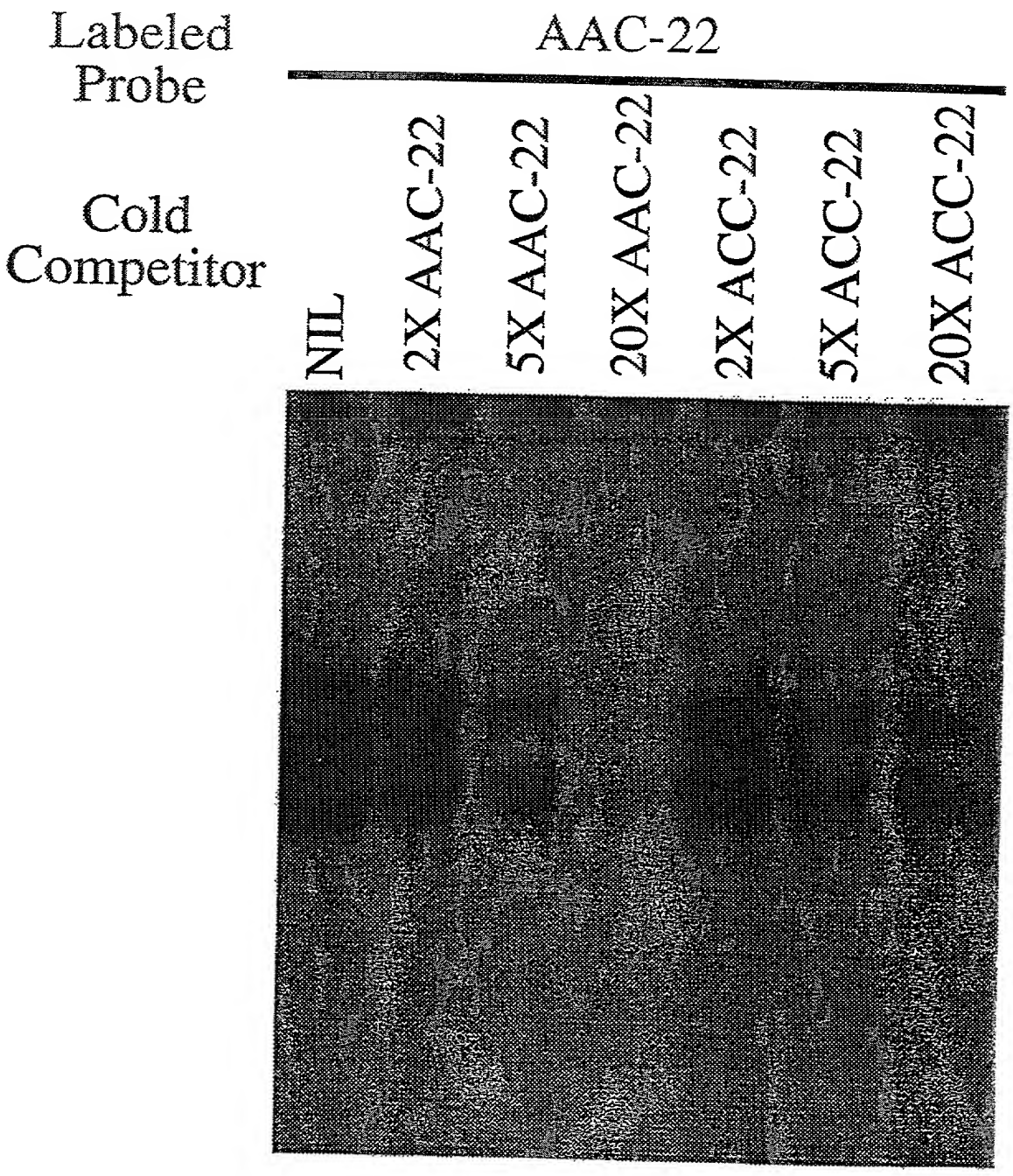

Labeled Probe
AAC-22
Cold Competitor
NIL
2X AAC-22
5X AAC-22
20X AAC-22
2X ACC-22
5X ACC-22
20X ACC-22

FIG. 7B

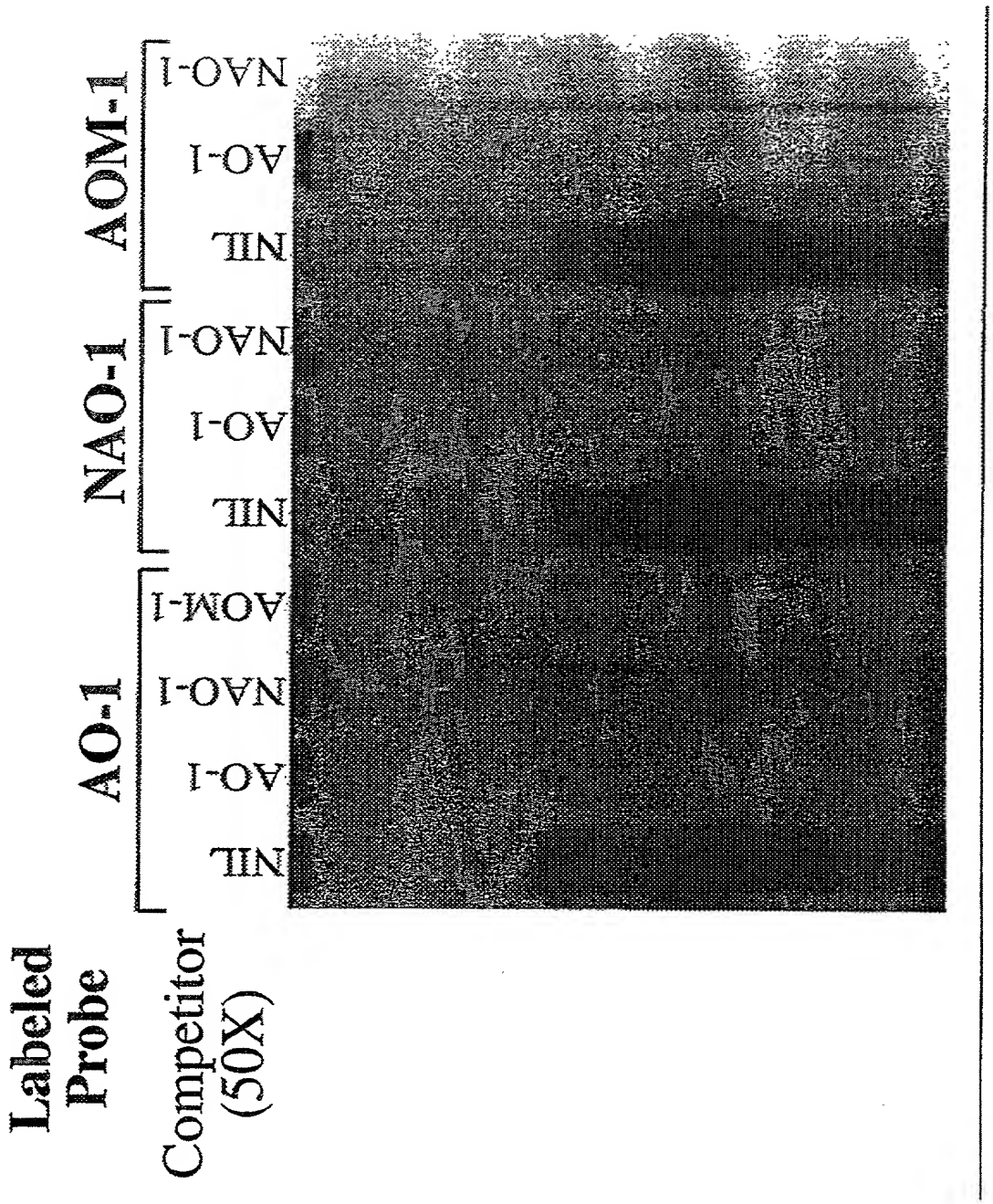

Labeled Probe
Competitor (50X)
AO-1
NIL
AO-1
NAO-1
AOM-1
NAO-1
AO-1
NIL
NAO-1
AO-1
NIL
AOM-1
NAO-1
AO-1
NIL

FIG. 7C

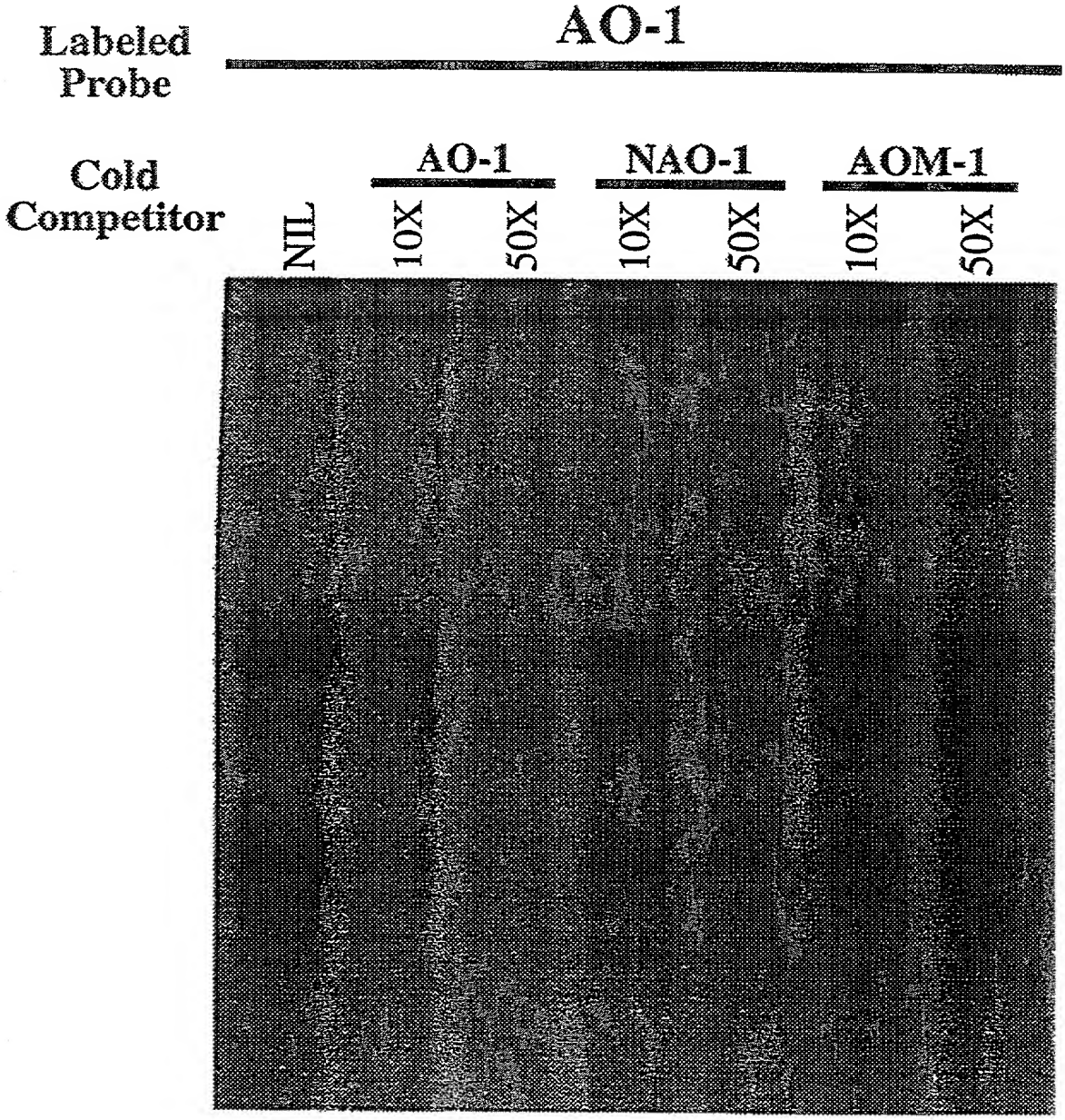

Labeled Probe
AO-1
Cold Competitor
AO-1
NAO-1
AOM-1
NIL
10X
50X
10X
50X
10X
50X

FIG. 7D

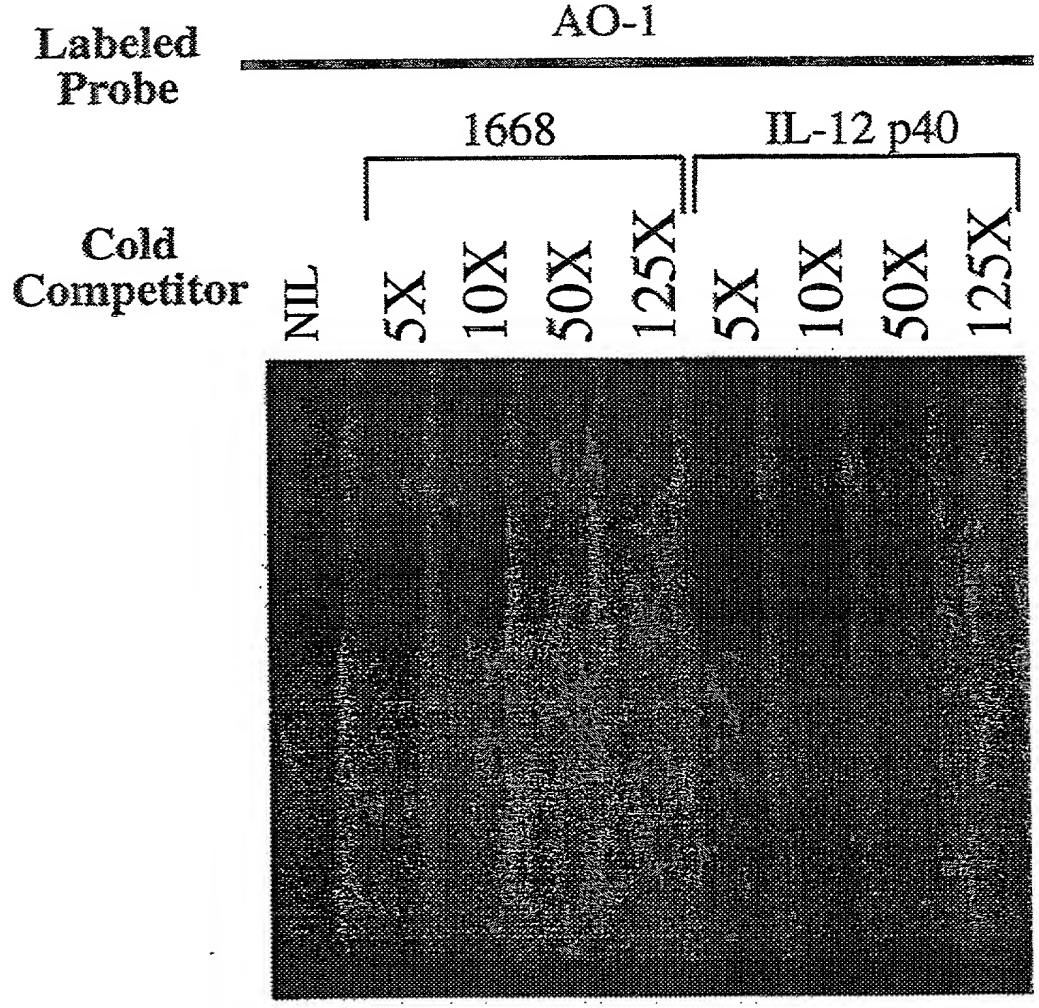

Labeled Probe
AO-1
1668
IL-12 p40
Cold Competitor
NIL
5X
10X
50X
125X
5X
10X
50X
125X

FIG. 7E

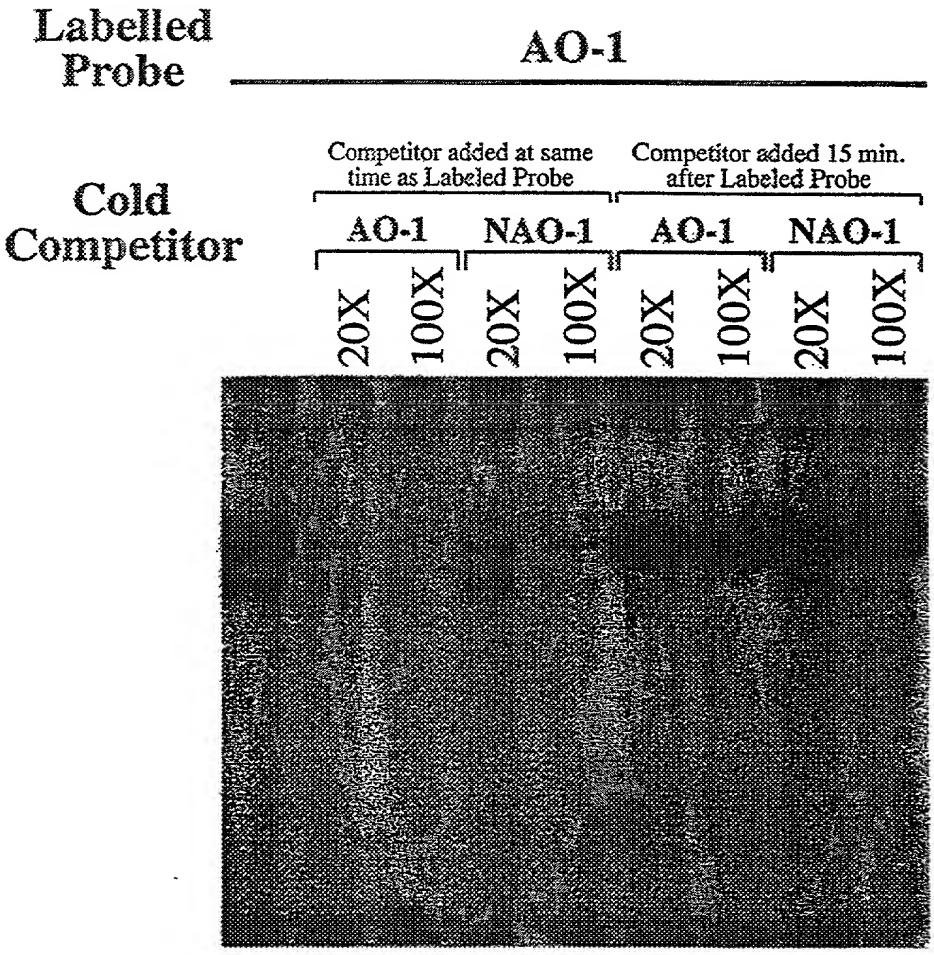

Labelled Probe
AO-1
Cold Competitor
Competitor added at same time as Labeled Probe
Competitor added 15 min. after Labeled Probe
AO-1
NAO-1
AO-1
NAO-1
20X
100X
20X
100X
20X
100X
20X
100X

FIG. 8A

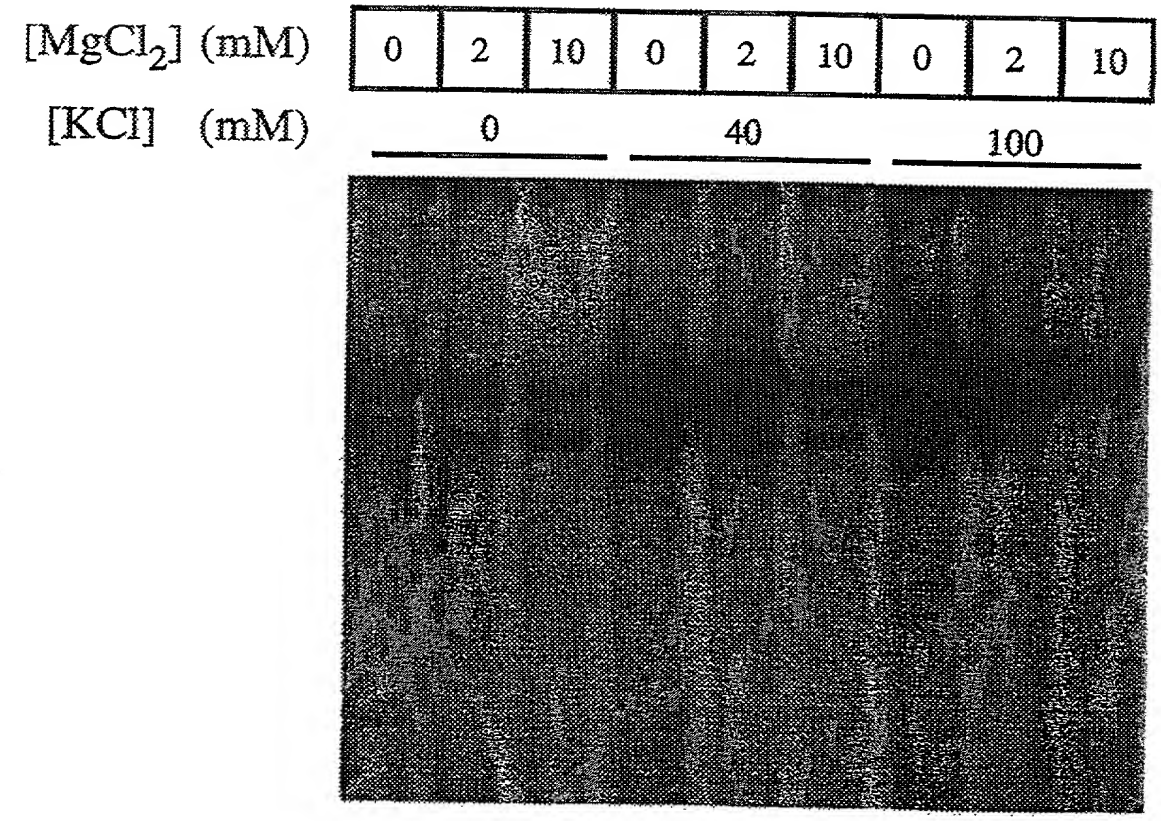


FIG. 8B

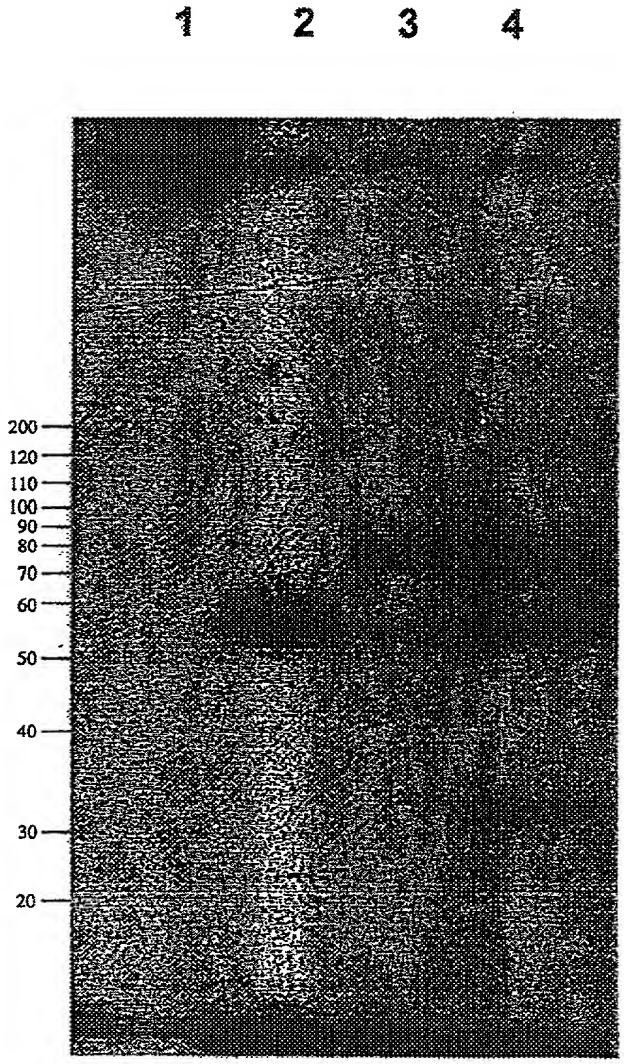


FIG. 9

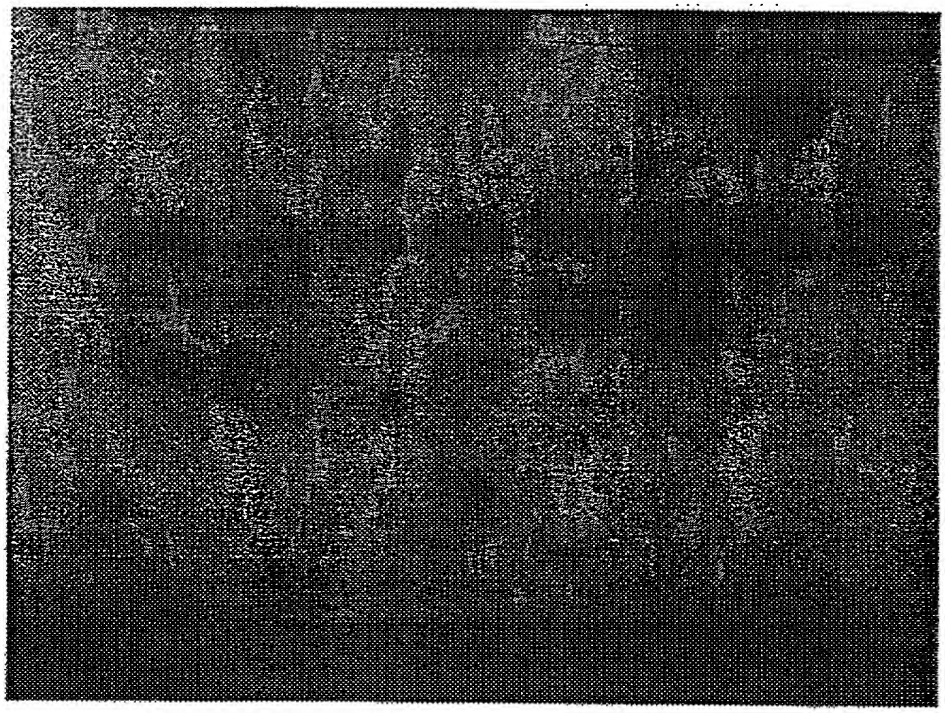

RAW-264.7
MonoMac 6
Free Probe
NIL
Control MAb
anti-p34 MAb
anti-p70MAb
NIL
Control MAb
anti-p34 MAb
anti-p70MAb

FIG. 10

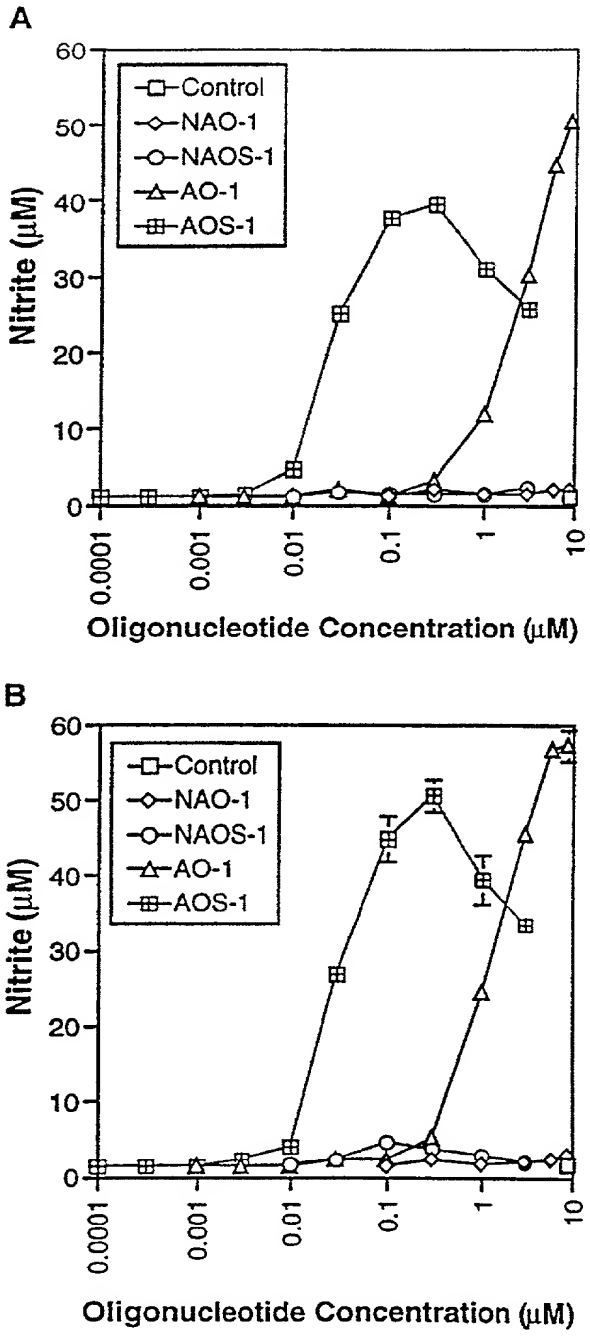

A
Nitrite (μM)
60
50
40
30
20
10
0
Control
NAO-1
NAOS-1
AO-1
AOS-1
0.0001
0.001
0.01
0.1
1
10
Oligonucleotide Concentration (μM)
B
Nitrite (μM)
60
50
40
30
20
10
0
Control
NAO-1
NAOS-1
AO-1
AOS-1
0.0001
0.001
0.01
0.1
1
10
Oligonucleotide Concentration (μM)

FIG. 11

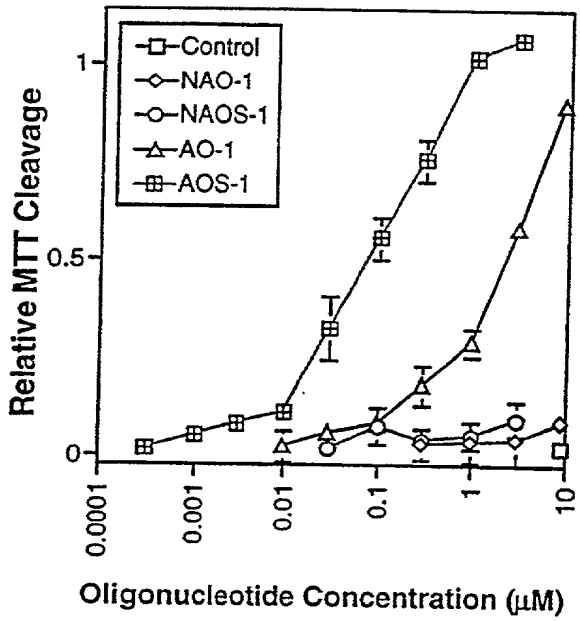

Control
NAO-1
NAOS-1
AO-1
AOS-1
Relative MTT Cleavage
0
0.5
1
0.0001
0.001
0.01
0.1
1
10
Oligonucleotide Concentration (μM)

FIG. 12

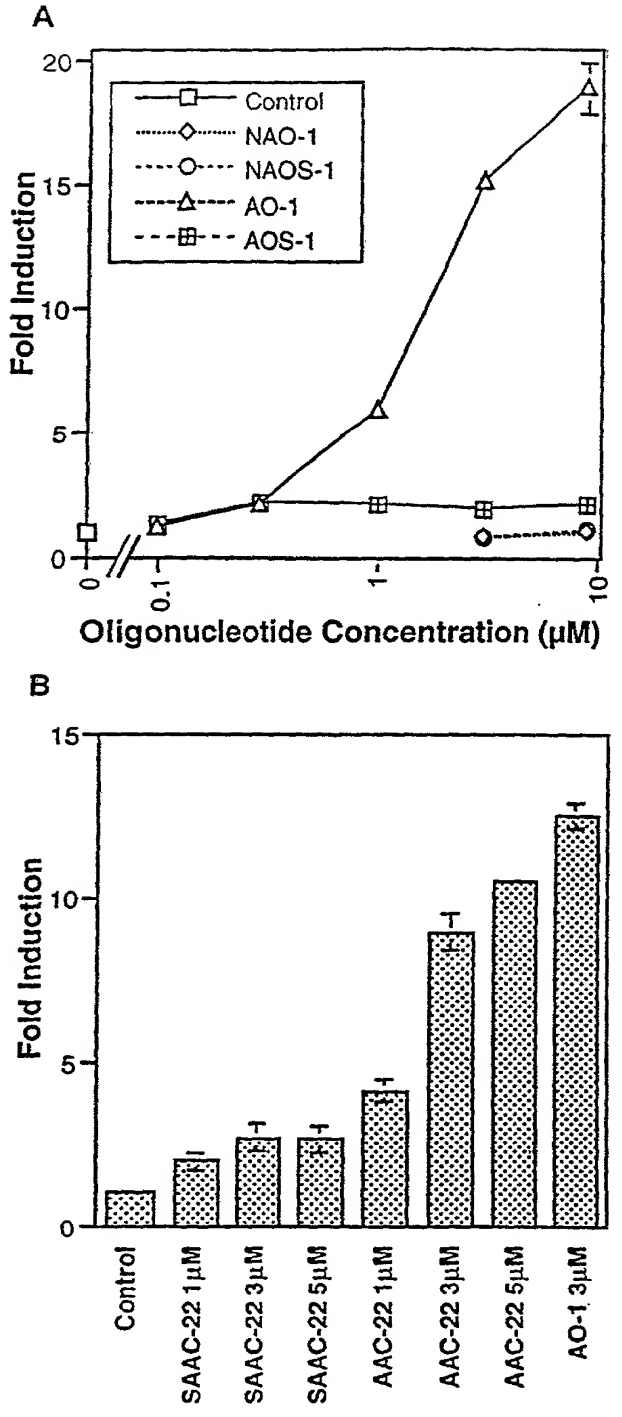

A
Control
NAO-1
NAOS-1
AO-1
AOS-1
Fold Induction
Oligonucleotide Concentration (μM)
B
Fold Induction
Control
SAAC-22 1μM
SAAC-22 3μM
SAAC-22 5μM
AAC-22 1μM
AAC-22 3μM
AAC-22 5μM
AO-1 3μM

FIG. 13

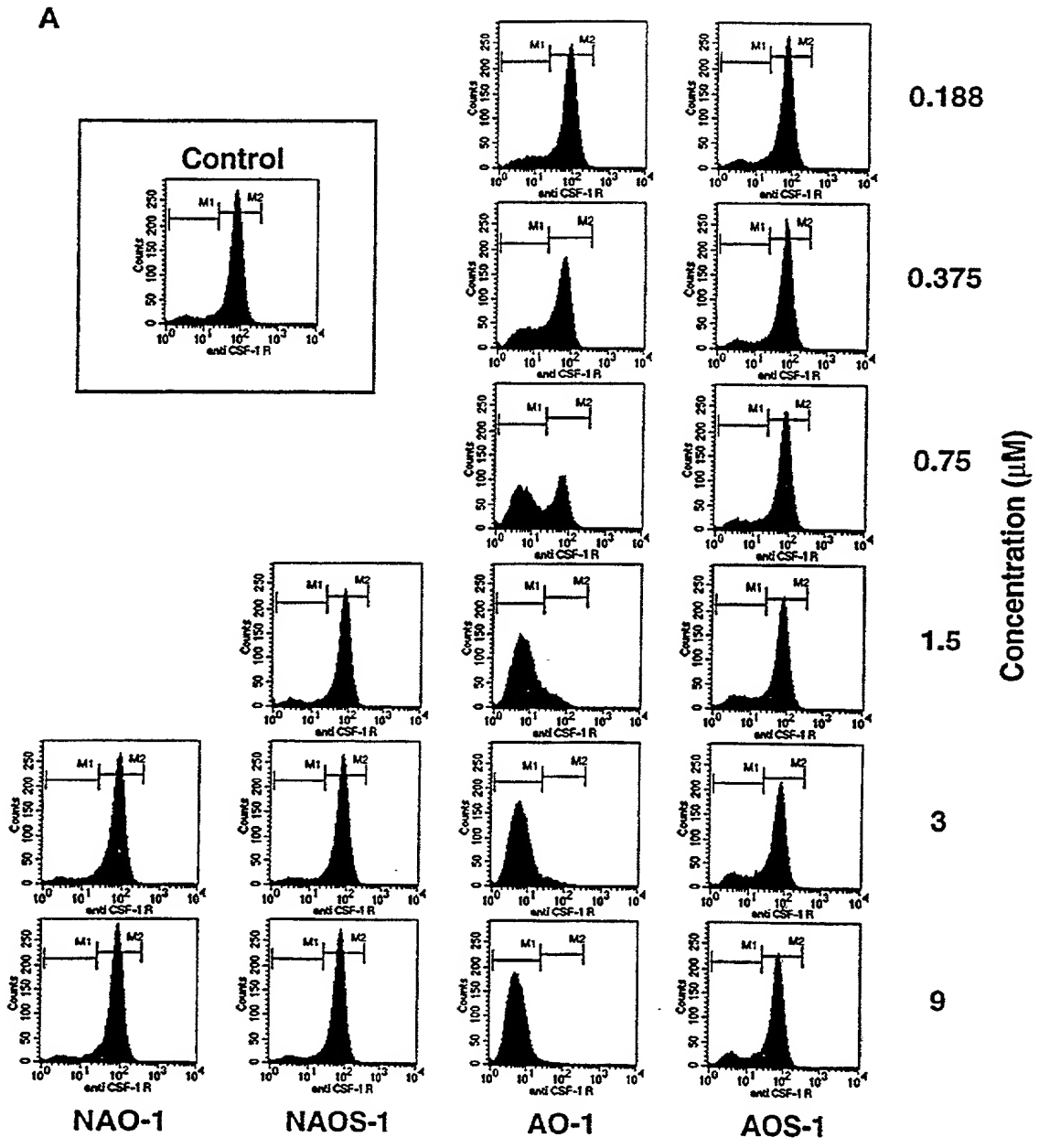
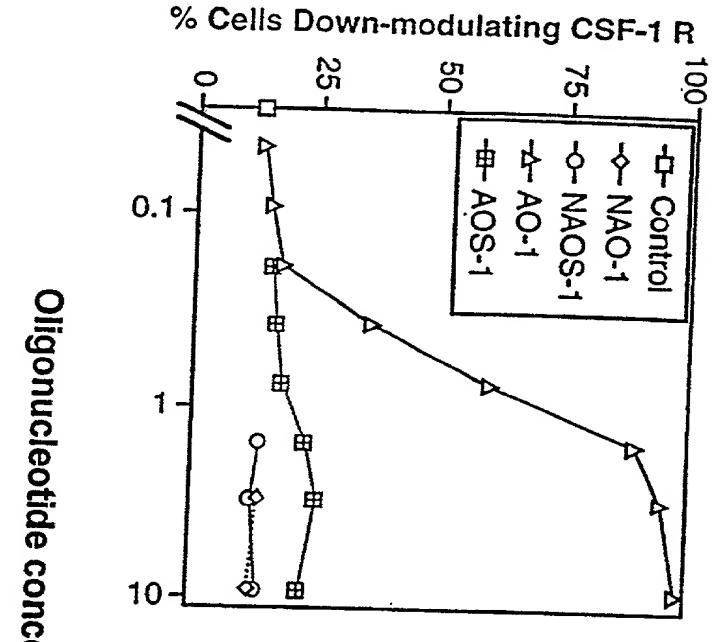
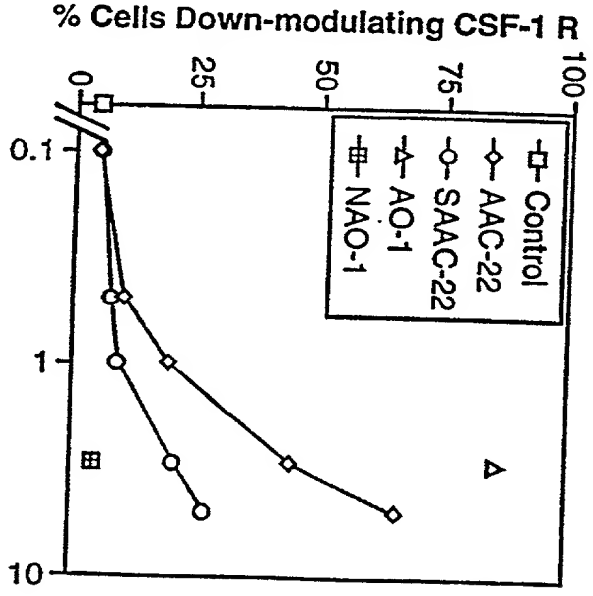

A
Control
0.188
0.375
0.75
1.5
3
9
Concentration (μM)
NAO-1
NAOS-1
AO-1
AOS-1
M1
M2
Counts
anti CSF-1 R

FIG. 14A

B



C



Oligonucleotide concentration (μM)

FIG. 14B&C

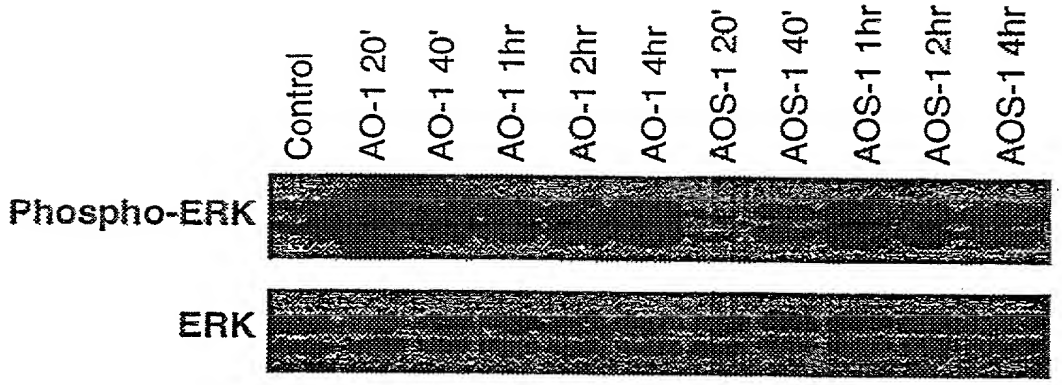
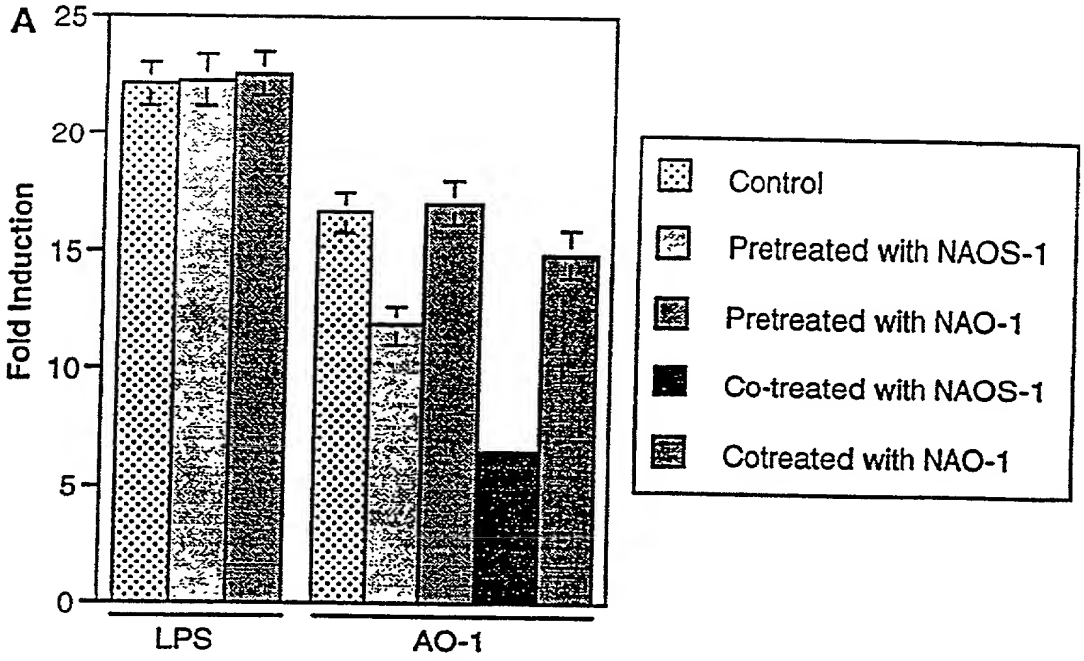

Control
AO-1 20'
AO-1 40'
AO-1 1hr
AO-1 2hr
AO-1 4hr
AOS-1 20'
AOS-1 40'
AOS-1 1hr
AOS-1 2hr
AOS-1 4hr
Phospho-ERK
ERK

FIG. 15


A
Fold Induction
25
20
15
10
5
0
LPS
AO-1
Control
Pretreated with NAOS-1
Pretreated with NAO-1
Co-treated with NAOS-1
Cotreated with NAO-1

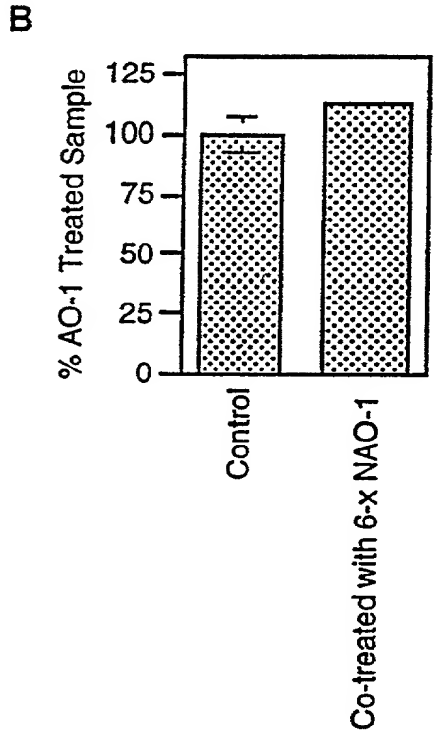

B
% AO-1 Treated Sample
125
100
75
50
25
0
Control
Co-treated with 6-x NAO-1

FIG. 16

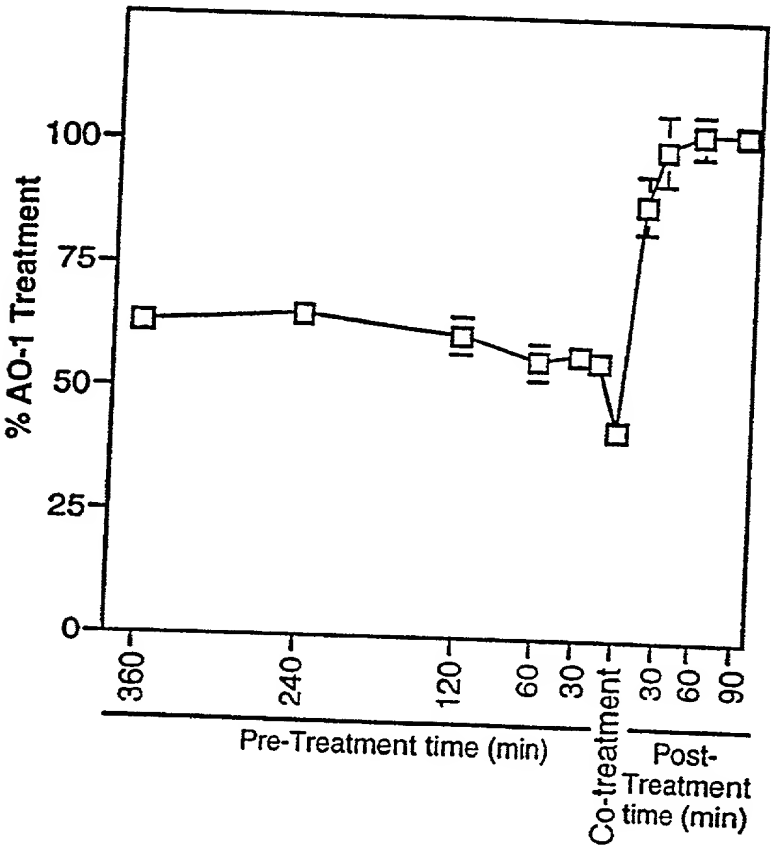
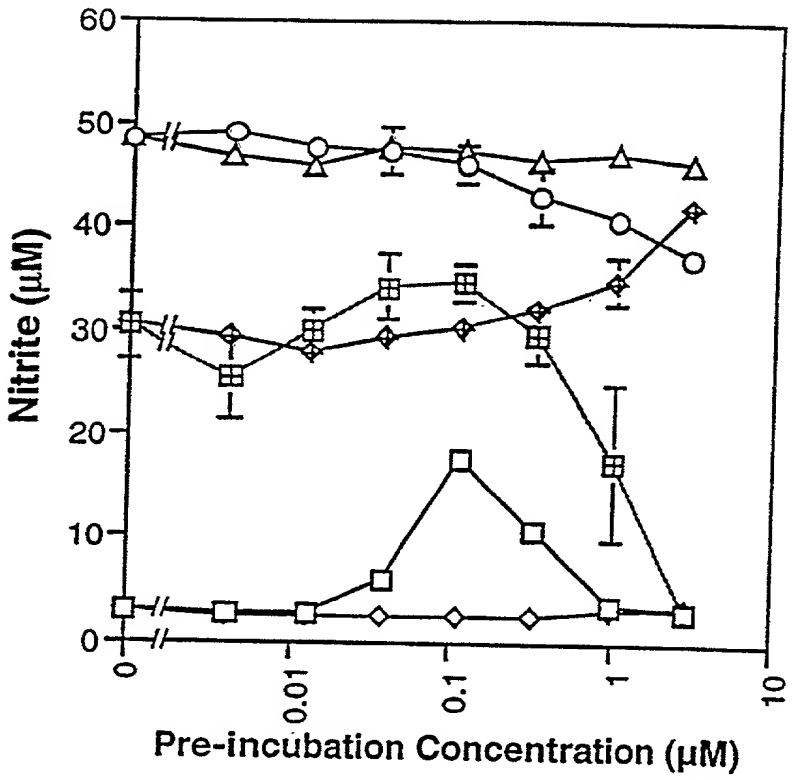

% AO-1 Treatment
100
75
50
25
0
360
240
120
60
30
30
60
90
Pre-Treatment time (min)
Co-treatment
Post-Treatment time (min)

FIG. 17


60
50
40
30
20
10
0
Nitrite (µM)
0
0.01
0.1
1
10
Pre-incubation Concentration (µM)

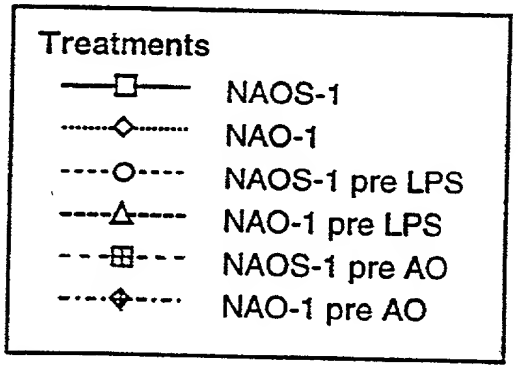

Treatments
NAOS-1
NAO-1
NAOS-1 pre LPS
NAO-1 pre LPS
NAOS-1 pre AO
NAO-1 pre AO

FIG. 18

FIG. 19A&B

A
Nitrite (μM)
0
20
40
60
0
0.1
1
10
100
IO-2 Co-treatment Concentration
Control
AO-1 1μM
IO-2 + AO-1 1 μM
B
Nitrite (μM)
0
10
20
30
40
50
0.1
1
10
100
Oligonucleotide Concentration
NAO-1
AO-1
IO-2

C
Nitrite (μM)
0
10
20
30
40
50
Control
IO-2
DNase I Treated IO-2
D
Nitrite (μM)
0
20
40
60
0
0.1
1
10
100
IO-2 Pre-incubation Concentration
Control
AO-1 1 μM
IO-2 2hr Pre-incubation/AO-1 1 μM
IO-2 6hr Pre-incubation/AO-1 1 μM

FIG. 19C&D

PTO/SB/01 (12/97)
Approved for use through 09/30/00. OMB 0651-0032
Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

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DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION

[X] Declaration Submitted with Initial Filing [ ] Declaration Submitted after Initial Filing

Attorney Docket	8191-PA01
First Named Inventor	Katryn Stacy et al.
COMPLETE IF KNOWN	
Application Number	Not Yet Assigned
Filing Date	
Group Art Unit	Not Yet Assigned
Examiner Name	Not Yet Assigned

As a below named Inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

IMMUNOSTIMULATORY DNA

(*Title of the Invention*)

the specification of which

[ ] is attached hereto

OR

[X] was filed on (MM/DD/YYYY) 11/25/1999 as United States Application Number or PCT International Application Number PCT/AU99/01052 and was amended on (MM/DD/YYYY) _____ (if applicable.)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37 Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Numbers	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	Certified Copy Attached? NO
PCT/AU99/01052	PCT	11/25/2000	[ ]	[ ]	[X]
PP 7288	Australia	11/25/1998	[ ]	[ ]	[X]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]

[ ] Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto:

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)

[ ] Additional provisional application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto

[DECESTAC01.E22]

DECLARATION - Utility or Design Patent Application

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s), or §365(c) of any PCT International application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior Unite d States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Patent Application Number	PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith. Registered practitioner(s) name/registration number listed below:

Name	Registration Number	Name	Registration Number
NEIL F. MARTIN JOHN L. HALLER JAMES W. MCCLAIN (3)	23,088 27,795 ~~24,536~~		

Direct all correspondence to:

Attorney Name	James W. McClain				
Address	BROWN MARTIN HALLER & McCLAIN LLP				
Address	1660 UNION STREET				
City	SAN DIEGO	State	CALIFORNIA	ZIP	92101
Country	USA	Telephone	(619) 238-0999	Fax	(619) 238-0062

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

NAME OF SOLE OR FIRST INVENTOR: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])				Last Name			
2.00 Katryn Jane				~~STACY~~ K.S.			
Inventor's Signature	Kate Stacy			Date 18/6/01			
Residence: City	St. Lucia	State		Country	Australia	Citizenship	AU AUX
Post Office Address	24 Highview Terrace						
Post Office Address							
City	St. Lucia,	State	Queensland	Zip	QLD 4067	Country	Australia

NAME OF SECOND INVENTOR: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])				Last Name			
4.00 David Peter				SESTER			
Inventor's Signature				Date 18 June 2001			
Residence: City	~~St. Lucia~~ FORRESTDALE	State	QLD	Country	Australia	Citizenship	AU AUX
Post Office Address	~~29 Hawken Drive~~ 39 PEIRWOLD CRT						
Post Office Address							
City	~~St. Lucia~~ FORRESTDALE	State	Queensland	Zip	QLD ~~4067~~ 4118	Country	Australia

☒ Additional Inventors are being named on the supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto.

[DECESTAC01.E22]

PTO/SB/02A (3-97)
Approved for use through 09/30/98. OMB 0651-0032
Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
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DECLARATION

ADDITIONAL INVENTOR(S)
Supplemental Sheet
Page 1 of 1

Name of Additional Joint Inventor, if any:	☐ A petition has been filed for this unsigned inventor						
Given Name (first and middle [if any])			Family Name or Surname				
1.00 Matthew James			SWEET				
Inventor's Signature	Matthew Sweet			Date 18/6/01			
Residence: City	~~Glasgow~~ BRISBANE	State QLD	Country ~~Great Britian~~ AUSTRALIA		Citizenship ~~GB~~ AUSTRALIAN	AUX	
Post Office Address	~~G1 21 Kirkland Stret~~ 3/105 SIR FRED SCHONELL DVE						
Post Office Address	~~North Kelvinside~~ ST. LUCIA						
City	~~Glasgow~~ BRISBANE	State ~~Strathclyde~~ QLD	Zip 4067	G20 6SY	Country	GB	
Name of Additional Joint Inventor, if any:	☐ A petition has been filed for this unsigned inventor						
Given Name (first and middle [if any])			Family Name or Surname				
2.00 David Arthur			HUME				
Inventor's Signature				Date	18-6-01		
Residence: City	Ashgrove	State QLD	Country Australia		Citizenship AU	AUX	
Post Office Address	27 Aloomba Road						
Post Office Address							
City	Ashgrove	State Queensland	Zip	QLD 4060	Country	Australia	
Name of Additional Joint Inventor, if any:	☐ A petition has been filed for this unsigned inventor						
Given Name (first and middle [if any])			Family Name or Surname				
Inventor's Signature				Date			
Residence: City		State	Country		Citizenship		
Post Office Address							
Post Office Address							
City		State	Zip		Country		

[DECESTAC01.E22]

SEQUENCE LISTING

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<120> IMMUNOSTIMULATORY DNA

<130> immunoDNA

<140> 09/856655
<141> 2001-05-24
<150> PCT/AU99/01052

<151> 1999-11-25

<150> PP7288

<151> 1998-11-25

<160> 14

<170> PatentIn Ver. 2.0

<210> 1

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

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oligonucleotide

<400> 1

gctcatgacg ttcctgatgc tg
22

<210> 2

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: AOM-1

oligonucleotide

<220>

<221> Modified_base
<222> (9)
<223> cm

<400> 2

gctcatgacg ttcctgatgc tg
22

<210> 3

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: NAO-1
oligonucleotide

<400> 3

gctcatgagc ttcctgatgc tg
22

<210> 4

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: AOS-1
oligonucleotide

<220>

<221> misc_feature

<222> (1)..(22)

<223> phosphorothioate

<400> 4

gctcatgacg ttcctgatgc tg
22

<210> 5

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: NAOS-1
oligonucleotide

<220>

<221> misc_feature

<222> (1)..(22)

<223> phosphorothioate

<400> 5

gctcatgagc ttcctgatgc tg
22

<210> 6

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: IO-2
oligonucleotide

<400> 6

gctcatgccg gtcctgatgc tg
22

<210> 7

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: AAC-22
oligonucleotide

<400> 7

accgataacg ttgccggtga cg
22

<210> 8

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: ACC-22 oligonucleotide

<400> 8

accgataccg gtgccggtga cg
22

<210> 9

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: M12 oligonucleotide

<400> 9

atccataacg ttccagaagc tg
22

<210> 10

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: PU-1
oligonucleotide

<400> 10

gtaggaccgg aagtgggagt
20

<210> 11

<211> 99

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Oct

oligonucleotide

<400> 11

aattcgagct cggtacccga tcctagcccc tctatgcaaa tgagaagcat tcctttcgaa
60
ttggggatcc tctagagtcg acctgcaggc atgcaagct
99

<210> 12

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: 1668
oligonucleotide

<400> 12

tccatgacgt tcctgatgct
20

<210> 13

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: IL-12 p40
oligonucleotide

<400> 13

gctatgacgt tccaaggg
18

<210> 14

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: SAAC-22
oligonucleotide

<220>

<221> misc_feature

<222> (1)..(22)

<223> phosphorothioate

<400> 14

accgataacg ttgccggtga cg
22

09/856655
JC18 Rec'd PCT/PTO 24 MAY 2001
PCT/AU99/01052

SEQUENCE LISTING

<110> THE UNIVERSITY OF QUEENSLAND

<120> IMMUNOSTIMULATORY DNA

<130> immunoDNA

<140>
<141>

<150> PP7288
<151> 1998-11-25

<160> 14

<170> PatentIn Ver. 2.0

<210> 1
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: AO-1
oligonucleotide

<400> 1
gctcatgacg ttcctgatgc tg 22

<210> 2
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: AOM-1
oligonucleotide

<220>
<221> modified_base
<222> (9)
<223> cm

<400> 2
gctcatgacg ttcctgatgc tg 22

<210> 3

<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: NAO-1
oligonucleotide

<400> 3
gctcatgagc ttcctgatgc tg 22

<210> 4
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: AOS-1
oligonucleotide

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